Investigating HIV-1 DNA and HIV-1 RNA kinetics with ultrasensitive assays in very early treated infants and determining predictors associated with kinetics in older treated children

by
Kirsten Abigail Veldsman

Thesis presented in fulfilment of the requirements for the degree of Master of Science in Medical Virology from the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Professor Gert Uves van Zyl

December 2019

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Full name : Kirsten Abigail Veldsman :

Date December 2019

Signature :

Abstract

Background

The impact of early initiation of antiretroviral therapy (ART) on HIV-1 persistence has been characterised in adult cohorts and in well-suppressed children on continuous therapy. These studies have shown that early ART initiation may limit the size of the latent HIV reservoir that is established early in infection. Early initiation of therapy causes a more rapid decay of infected cells and may increase the probability of post-treatment control which is valuable for HIV-1 remission strategies. However, there remains limited information from longitudinal studies from resource limited settings especially investigating the effects of therapy initiation within days of birth on HIV-1 DNA and viral decay kinetics. Our aims were to investigate total HIV-1 DNA and HIV-1 RNA decay in very early treated infants and in older treated children who underwent therapy interruption and to determine potential predictors influencing decay.

Methods

Participants were selected from two cohorts; eleven infants from a public health sector birth diagnosis program who initiated ART at median of four days and 31 children from a clinical trial study where children were randomised to elective time-limited therapy or delayed continuous therapy. Peripheral blood mononuclear cells (PBMCs) were processed at scheduled study visits and plasma viral load measured using commercial diagnostic assays. Following DNA extraction from PBMCs, total HIV-1 DNA was quantified using a sensitive real-time PCR assay adapted for HIV-1 subtype C targeting a conserved region in the HIV-1 genome (integrase gene). Generalised linear and mixed effect regression models; and Spearman rank correlations were used to study decay and associating predictors. Statistical tests were implemented in R software version 3.4.3.

Results

In our study we observed that infants who initiated therapy at around five days had a faster decay rate than children who initiated ART at around five months of age, the half-life (t ½) HIV-1 DNA was 2.7 months and 9.2 months, respectively. In the multivariate model, high pre-treatment HIV-1 DNA level ($p<0.001$) and an increase in HIV-1 DNA concentration during the period of therapy interruption ($p<0.01$) were independent significant predictors of slower subsequent HIV-1 DNA decay. In contrast, children who received prolonged initial treatment for 96 weeks had a faster decay after reinitiating on therapy ($p=0.02$).

Conclusion

This study provided some of the first longitudinal data of HIV-1 DNA decay in children from a resource limited setting. We suggest early treatment as an important modifiable factor in determining HIV-1 DNA decay. Furthermore, we show that very early diagnosis and subsequent therapy initiation, when achieving adequate virological suppression, in the early stages of infection may be valuable in limiting the persistence of long-lived HIV-1 infected cells.

Opsomming

Agtergrond

Die impak van vroeë inisiasie van antiretrovirale terapie (ART) op MIV-1-voortbestaan is gekenmerk in volwasse kohorte en in goed-onderdrukte kinders tydens volgehoue terapie. Hierdie studies het getoon dat vroeë ART-aanvang die grootte van die latente MIV-reservoir wat vroeg in infeksie gevestig word, kan beperk. Vroeë terapie aanvang veroorsaak 'n vinniger verval van geïnfekteerde selle en kan die waarskynlikheid van na-behandeling beheer, wat waardevol is vir MIV-1 remissie strategieë, verhoog. Daar bly egter beperkte inligting van longitudinale studies van hulpbronbeperkte instellings, veral om die gevolge van terapieinisiëring binne dae van geboorte op MIV-1 DNs en virale vervalkinetika te ondersoek. Ons doelwitte was om die totale MIV-1 DNS en MIV-1 RNS verval in baie vroeë behandelde babas en in ouer behandelde kinders wat terapie onderbreek het, te ondersoek en om te bepaal die potensiële voorspellers wat die verwal beïnvloed.

Metodes

Deelnemers is gekies uit twee kohorte; elf babas van 'n geboortediagnose-program vir openbare gesondheidsorg wat ART begin het teen `n mediaan van vier dae en 31 kinders van 'n kliniese proefstudie waar kinders gerandomiseer is vir elektiewe tydbeperkende terapie of uitgestelde volgehoue terapie. Perifere bloed mononukleêre selle (PBMS's) is geprosesser tydens geskeduleerde studiebesoeke en plasma virale lading gemeet met kommersiële diagnostiese toetse. Na DNA-ekstraksie van PBMS's, is die totale MIV-1 DNA gekwantifiseer met behulp van 'n sensitiewe real-time PKR-bepaling aangepas vir MIV-1 subtipe C wat 'n min-varierende gebied in die MIV-1-genoom (integrasegeen) teiken. Algemene lineêre en gemengde effek regressie modelle; en Spearman rangkorrelasies is gebruik om verval te bestudeer en voorspellers te assosieer. Statistiese toetse is geïmplementeer in R sagteware weergawe 3.4.3.

Resultate

In ons studie het ons opgemerk dat babas wat terapie teen ongeveer vier dae begin het, 'n vinniger verval gehad het as kinders wat ART teen ongeveer vyf maande begin het. Die MIV-1 DNA halfleeftyd (t ½) was onderskeidelik, 2.7 maande en 9.2 maande. In die multivariate model was hoë voor-behandeling MIV-1 DNA vlak ($p < 0.001$) en 'n toename in MIV-1 DNA konsentrasie gedurende die periode van terapie onderbreking ($p < 0.01$) onafhanklike belangrike voorspellers van stadiger daaropvolgende MIV-1 DNA verval. In teenstelling hiermee, het kinders wat langdurige aanvanklike behandeling gedurende 96 weke ontvang het, 'n vinniger verval ná terapie re-inisiasie ($p = 0,02$).

Gevolgtrekking

Hierdie studie het van die eerste longitudinale data van MIV-1 DNA-verval in kinders uit 'n hulpbron beperkte omgewing gelewer. Ons stel vroeë behandeling as 'n belangrike wysigbare faktor in die bepaling van MIV-1 DNA-verval voor. Verder wys ons dat baie vroeë diagnose en die daaropvolgende terapie-aanvang met voldoende virale onderdrukking, in die vroeë stadiums van infeksie, waardevol kan wees om die voortbestaan langlewende MIV-1-geïnfekteerde selle te beperk.

Acknowledgments

I would like to thank the following individuals and groups:

I. Professor Gert van Zyl for his guidance and mentorship. The level of support I received is truly appreciated and will always be remembered. I have learnt many lessons from his style of supervision and leadership; and valuable lessons from his remarkable career as a physician scientist.

II. Research team members (past and present):
   - Shahieda Isaacs for assay training and always assisting me when needed.
   - Mary Grace Katusiime for assay training.
   - Heleen le Grange for assay training.
   - Ian Botha, Carli van Zyl and Kayla Delaney for support in the form of research advice, encouraging words, humour and delicious food.

III. Research team at KIDCRU at Tygerberg Hospital for sample collection and assistance with retrieval of clinical data. I would like to especially thank Professor Mark Cotton for his valuable input on conference proceedings and manuscripts submitted for publication. His input has helped guide my thinking as I wrote my thesis.

IV. Dr Carl Lombard for his assistance with the statistical analyses of my research project.

V. Our research collaborators in the United States of America for their expert advice on the work related to this research project. Their input helped steer this project into a positive direction with good outcomes.

VI. Students and staff of the Division of Medical Virology for their support especially Karmistha Poovan and Shalena Naidoo, for the immense kindness they have shown me.

VII. Dolores, Caleb and Tess Veldsman for their continual love, support, guidance and prayers throughout this entire journey.

Last, but certainly not least, God (The Almighty, loving father and best friend) for everything is in Him and everything is possible through Him.

Dedication

I dedicate this thesis to:

My mother, **Dolores Jemane Veldsman,** who stood by me and walked this journey with me. We celebrated the times of success together and when challenges arose you were there as pillar of strength. A pillar made from wisdom and life's truths rooted in your love and kindness. Thank you for your fearless love, your teachings, your discipline and kindness.

I also dedicate this work in loving memory of **Matthew Veldsman**, an exceptional young man who left our family so soon. I have not fully come to terms with your passing because when I think about you, I think about my brother and the pain I feel is real. There are still many unanswered questions regarding your passing and this reminds me of the very reason that led me into the field of medical science. Perhaps one day we will fully understand, but for now we will celebrate your life and try to emulate your good qualities. May your soul rest in peace and I look forward to meeting you again.

Research Outputs

Below is a list of research outputs based on the work completed in this Master's project.

Publications:

Veldsman, K.A., Maritz, J., Isaacs, S., Katusiime, M., Janse van Rensburg, A., Laughton, B., Mellors, J.W., Cotton, M.F. & van Zyl G.U. 2018. **Rapid decline of HIV-1 DNA and RNA in infants starting very early ART may pose a diagnostic challenge**. *AIDS.* 32(5): 629-634.

Veldsman, K.A., Janse van Rensburg, A., Isaacs, S., Naidoo, S., Laughton, B., Lombard, C., Cotton, M.F., Mellors, J.W. & van Zyl G.U. 2019. **HIV-1 DNA decay is faster in children who initiate ART shortly after birth than later**. *JIAS* [submitted, in process of being reviewed]

International Conference attendance:

Oral presentation:

Veldsman, K.A., Maritz, J., Isaacs, S., Katusiime, M., la Grange, H., Janse van Rensburg, A., Laughton, B., Mellors. J.W., Cotton, M.F. & van Zyl, G.U. 2017. **Rapid Decline of Total HIV DNA in infants Starting ART within 8 Days of Birth.** *Conference on Retroviruses and Opportunistic Infections (CROI)*, Seattle, USA

Poster presentation:

Veldsman, K.A., Janse van Rensburg, A., Isaacs, S., Naidoo, S., Laughton, B., Lombard, C., Cotton, M.F., Mellors, J.W. & van Zyl, G.U. 2019. **Early ART start in children is associated with more rapid decay of HIV-1 DNA.** *Conference on Retroviruses and Opportunistic Infections (CROI)*, Seattle, USA

Table of Contents

List of Abbreviations

3TC	Lamivudine
ABC	Abacavir
AHI	Acute HIV infection
AIDS	Acquired Immunodefiency syndrome
ART	Antiretroviral therapy
AZT	Zidovudine
CAD	Cell associated DNA
CAP/CTM	(Roche) Cobas® AmpliPrep/Cobas® TaqMan® HIV-1 test v2.0
cART	Combination antiretroviral therapy
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4 (glycoprotein)
CHER	Children with HIV early antiretroviral therapy
CHI	Chronic HIV Infection
CTLs	Cytolytic T lymphocytes
D4T	Stavudine
DBS	Dried blood spot
DDI	Didanosine
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
DMSO	Dimethly sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EID	Early infant diagnosis
E-value	Efficiency value
FBS	Fetal bovine serum
Gly	Glycogen
GuHCl	Guanidinium hydrochloride

GuSCN	Guanidinium isothiocyanate
HIV	Human Immunodeficiency virus
iCAD	Integrase cell associated total HIV-1 DNA quantitative assay
LPV/r	Lopinavir-ritonavir
LRAs	Latency reversing agents
NHLS	National Health Laboratory Services
NRTI	Nucleoside or nucleotide reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PHI	Primary HIV infection
PMTCT	Prevention of mother-to-child transmission
ProK	Proteinase K
qPCR	Quantitative polymerase chain reaction (same as real-time PCR)
R^2	Correlation of coefficient
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (media)
t ½	Half-life
Tris-HCl	Trisaminomethane hydrochloride
VEID	Very early infant diagnosis
VOA	Viral outgrowth assay

Table of Figures

Table of Tables

Chapter 1: Introduction (Literature Review)

1.1 The HIV research arena

The United Nations (UN) member states have pledged their support to end the AIDS epidemic by the year 2030. To make this goal achievable the UN has established the 90-90-90 treatment target as the major initiative to guide countries towards ending the epidemic in their region. The 90-90-90 treatment target stipulates that by the year 2020, of all people infected with HIV 90% should know their status, 90% of all HIV infected should be receiving sustained antiretroviral therapy (ART) and 90% of all receiving ART should reach viral suppression (Joint United Nations Program on HIV/AIDS, 2014). Although countries are making steady progress towards achieving this target there remains a large proportion of individuals on ART regimens or needing access to ART. Lifelong ART comes with many side effects for the patient and it is economically not sustainable for health sectors to maintain (Mikkelsen *et al.,* 2017). Therefore developing a cure for HIV remains a priority research area. Other research areas such as vaccine development and drug resistance remain an important focus in HIV research and ultimately the information generated from different disciplines all contribute towards ending the epidemic. This research project aims to contribute to knowledge related to HIV-1 persistence and HIV-1 remission (Figure 1.1) with a focus on paediatric HIV infection.

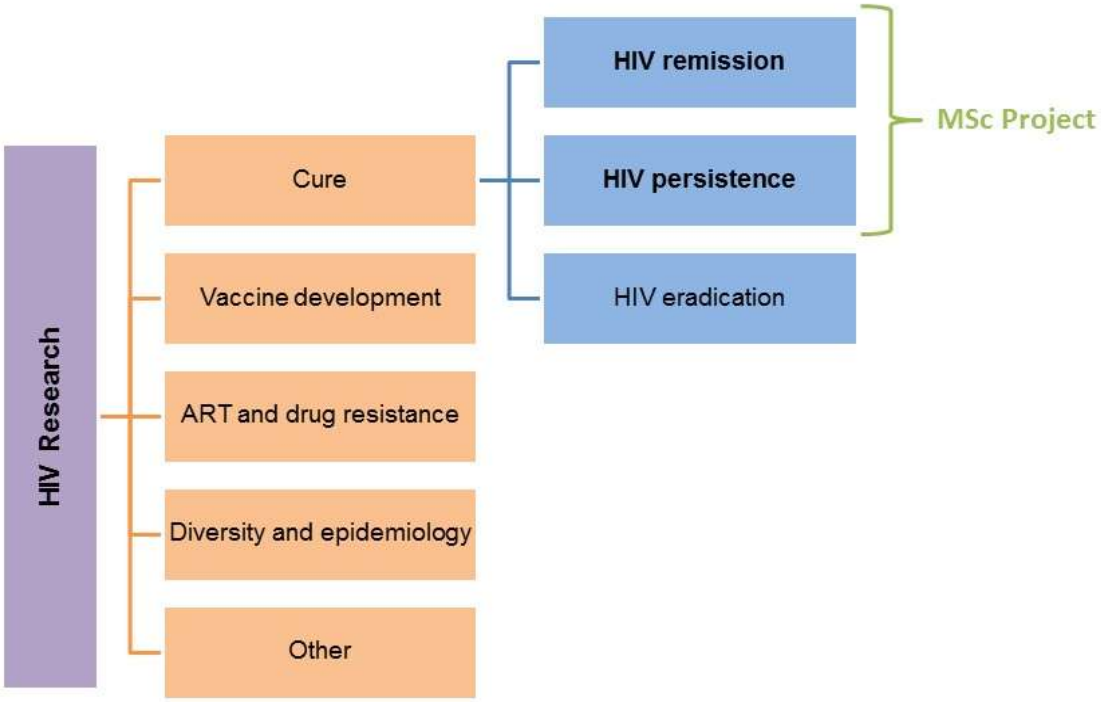


Figure 1.1 General overview of the areas of research related to HIV.

1.2 Worldwide HIV burden

The latest global HIV statistics report an estimate of 36.9 million people living with HIV with 21.7 million having access to antiretroviral therapy (ART). Eastern and Southern Africa remain the regions of the world most affected by the HIV pandemic with 19.6 million HIV positive infected individuals of which 12.9 million have access to ART (UNAIDS, 2018). South Africa carries the largest burden of the disease globally with an estimated 7.2 million infected individuals with a prevalence of approximately 12.6% (Statistics South Africa, 2017). Globally there are approximately 1.8 million children aged between 0 and 14 years living with HIV, the large majority are from Eastern and Southern Africa (an estimated 1.2 million).

Attempts to reduce HIV transmission through interventions is complicated by the highest burden of HIV infections being in resource limited regions. Despite the on-going success of increasing the availability of ART to all regions affected by the pandemic, HIV incidence is declining too slowly to achieve control (Bekker *et al.,* 2018). Along with the drive to improve access to ART to all infected individuals, a focus on implementing primary HIV preventative strategies are needed to accelerate the decline in the rate of new infections.

1.3 Perinatal transmission and Paediatric HIV

1.3.1 Prevention of mother-to-child HIV-1 transmission

The majority of paediatric infections occur as a result of mother to child transmission either during pregnancy (in utero), during delivery (intrapartum) or through breastfeeding. The rate of transmission has been dramatically reduced since the implementation of mother-to-child therapy programs (Luzuriaga & Mofenson, 2016). In early trials, such as the Paediatric AIDS Clinical Trials Group Protocol 076, researchers investigated the use of a single antiretroviral drug (ARV), zidovudine (AZT), given during pregnancy, labour and to the new born as a preventative strategy to prevent HIV-1 transmission to the child. The rate of transmission dropped to nearly 70% in infants who were not breast-fed (Connor *et al.*, 1994). Further studies assessed the use of two or three ARVs and showed an even greater reduction in transmission (Cooper *et al.,* 2002). Based on the evidence from these studies prevention of mother-to-child transmission (PMTCT) programs have been established in most regions affected by the pandemic. Guidelines have been set by the World Health Organisation for the implementation of PMTCT programs and the current recommendation is to initiate lifelong ART in all pregnant and breastfeeding women regardless of clinical stage or CD4 cell count (WHO, 2015). This option is known as option B+ and is considered the most beneficial strategy to prevent transmission in regions with a high HIV prevalence and high fertility rates (WHO, 2015). In 2017 approximately 93% of pregnant women in eastern and southern Africa where on ARV for the prevention of mother-to-child transmission and a total of 160,000 new infections were averted as a result of PMTCT programs (UNAIDS, 2018). In a recent

study, zidovudine-based ART or tenofovir-based ART had a lower transmission rate than zidovudine alone during pregnancy and single-dose nevirapine to the mother and baby. However, there were adverse maternal and neonatal outcomes associated with antenatal ART (Fowler *et al.,* 2017). Despite the potential risks associated with PMTCT programs, it most importantly has the overall benefit of reducing transmission and limiting disease progression and the risk of death in perinatally-infected children.

1.3.2 Characteristics of paediatric HIV-1 infection

HIV-1 disease progression in children is rapid and associated with a high mortality. In the absence of treatment infants frequently have plasma HIV RNA levels greater than 100 000 copies per millilitre for months after infection followed by a slow rate of decline reaching set-point at around five years of age in children who do not rapidly progress to death (Tobin & Aldrovandi, 2013). In contrast, in the absence of treatment adults have a much slower disease progression where AIDS or death only occurs at a median of ten years after acute infection. After acute infection the plasma HIV RNA level peaks, then it declines (up to a 1000-fold decrease) and a set-point is reached within weeks of infection and maintained (Martinez *et al.,* 2016). The immature immune system in infants with deficient HIV-1 specific CD4+ T cell responses, ineffective CD8+ T cell responses and a delay in antibody-dependent cell-mediated cytotoxicity may contribute to the lack of viraemic control observed in children (Martinez *et al.*, 2016; Tobin & Aldrovandi, 2013). Furthermore, microbial translocation has also been shown to be a major contributor to infant HIV-1 pathogenesis. The gastrointestinal tract has a large concentration of CD4+ CCR5+ T cells which are highly susceptible to HIV-1 infection (Roider *et al.,* 2016).

In the absence of ART HIV-1 disease progression in infants is much faster than in adults. However, when children are diagnosed shortly after birth it offers a unique opportunity to treat very early with ART. Moreover, the reservoir may be more labile in children with subsequent decay of infected cells to low levels as observed in the Mississippi baby and others. Early therapy may also preserve immune function which would be valuable for future immunotherapeutic interventions (Goulder *et al.,* 2016).

1.4 HIV-1 Persistence

1.4.1 HIV-1 integration and latency

HIV-1 persistence is a result of HIV-1 latency and in order to understand persistence one has to understand how latency is established and maintained. One of the characteristic features of all retroviruses is the ability to integrate double stranded viral DNA generated by reverse transcription of the viral RNA genome, into the host cell DNA (Coffin *et al.,* 1997). These integrated viral genomes are referred to as proviruses. The integration of the viral HIV-1 DNA into the host

genome is mediated by the integrase enzyme. Integration takes place at the termini of viral DNA, but integration into the host cell DNA can occur at various regions within the genome. Proviral DNA is copied when the host cell undergoes cell division and infectious viruses are produced when the provirus is transcribed and viral proteins are translated (Craigie & Bushman, 2012). Following integration, in some cells HIV-1 can establish a state of latent infection which enables escape from host immune surveillance (Siliciano & Greene, 2011). The establishment of the latent viral reservoir remains the main obstacle to achieving an HIV cure. How the latent reservoir is established is continually being investigated, but figure 1.2 provides an overview of the most likely explanation on how the latent reservoir for HIV-1 one is formed and maintained (Sengupta & Siliciano, 2018). Upon exposure to HIV-1, activated CD4+ T cells die as a result of the cytopathic effects of the virus or host immune responses which result in apoptosis. However, a subset of CD4+ T cells revert back to a resting state and HIV-1 gene expression is silenced because it requires the host transcription factors to continue the viral replication cycle. HIV-1 remains hidden in these memory cells and rapid viral rebound can occur in response to re-exposure to an antigen (Siliciano & Greene, 2011).

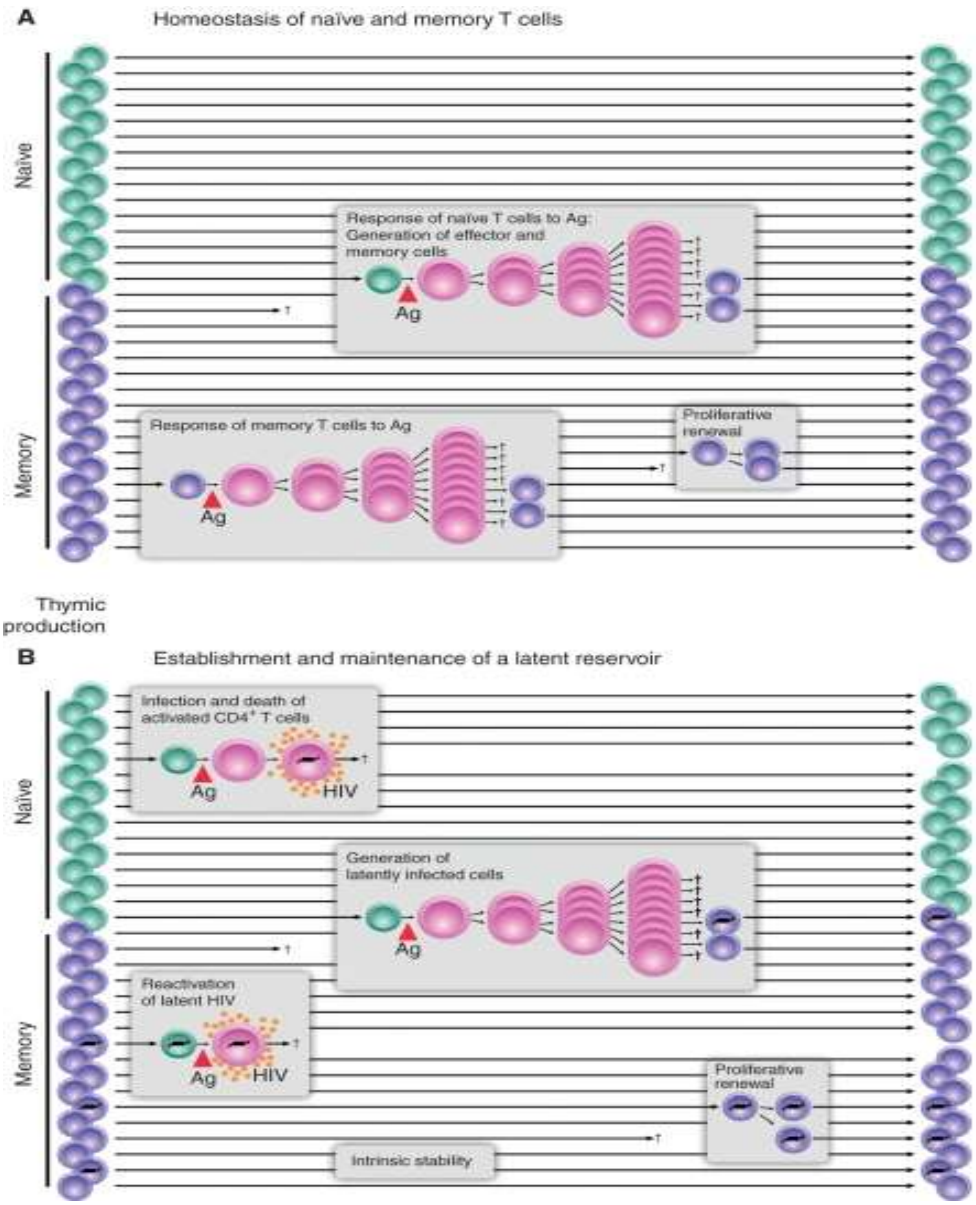


Figure 1.2 The establishment and maintenance of the latent HIV-1 reservoir. (Source: Siliciano & Greene, 2011)

1.4.2 HIV-1 replication and antiretroviral therapy

As shown in figure 1.3, the first step of the HIV-1 replication cycle is viral entry. HIV-1 fuses with the host CD4+ cell surface via recognition of CD4+ receptors and chemokine receptors CCR5 or CXCR4. HIV-1 envelope glycoprotein 120 (gp120) binds to CD4 receptor and the co-receptors and HIV-1 glycoprotein 41 (gp41) mediates fusion. After fusion with the host cell, HIV-1 RNA is converted through a few steps into double stranded DNA, which as pre-integration complex is transported across the nucleus and through the catalytic action of the integrase enzyme, it is integrated into the host genome, where it exists as a provirus. The host cellular components such as transcription factors are used to transcribe new viral RNA from the integrated proviral DNA. The new viral RNA contains the necessary information to make new viral proteins to assemble a new

virus. The new viral RNA and viral proteins assemble at the cell surface of the host cell to bud off and form an immature HIV virion. The protease enzyme initiates proteolysis to cleave viral polyproteins to create a mature infectious virion (Arts & Hazuda, 2012).

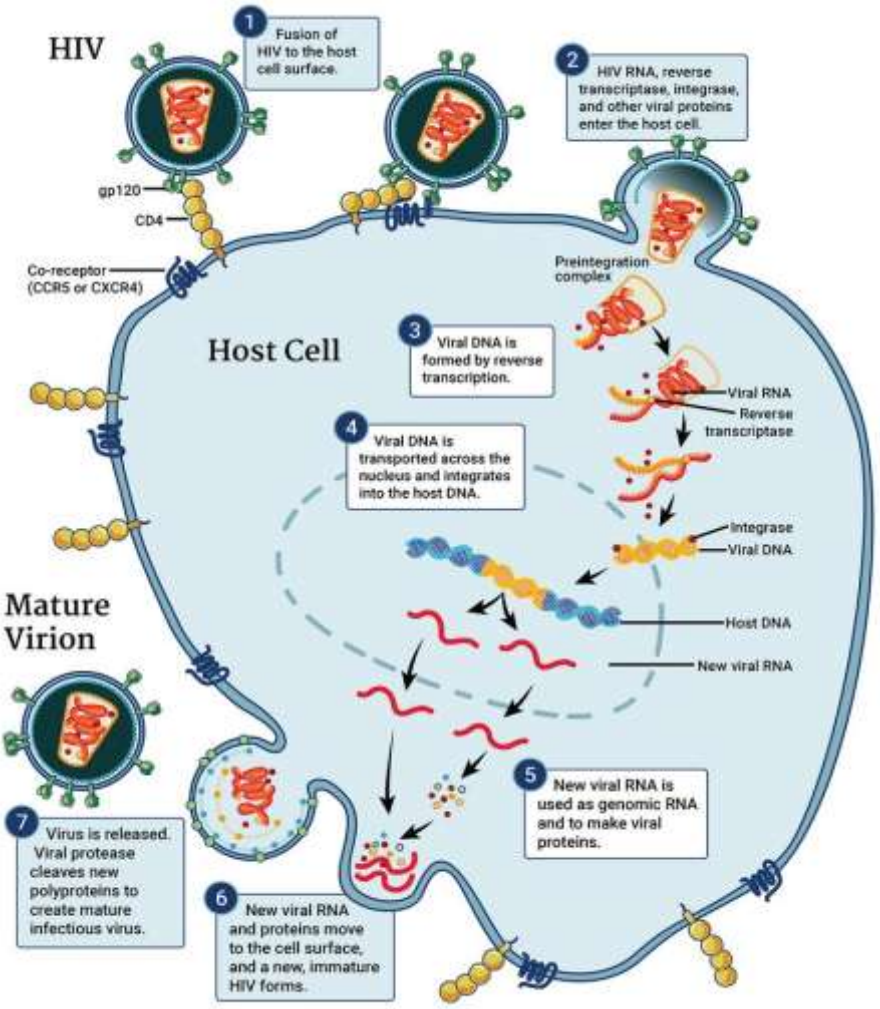


Figure 1.3 HIV-1 replication cycle (Source: National Institute of Allergy and Infectious Diseases, 2018. [Online]: https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle).

The understanding of the HIV-1 replication cycle has led to the development of antiretroviral drugs that effectively block different stages in the replication cycle. There are six classes of antiretroviral drugs that have been developed; nucleoside or nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase inhibitors, protease inhibitors, fusion inhibitors and co-receptor antagonists (Arts & Hazuda, 2012). Figure 1.4 shows where these respective classes of drugs inhibit the HIV-1 replication cycle. Antiretroviral therapy is provided in a combination of drugs, which have different mechanisms of action or compete with different substrates. Combination ART (cART) effectively blocks new rounds of infection of cells, reducing plasma HIV-1 viral load to undetectable levels on commercial assays, which is associated with immunological and clinical improvement. Despite the success of ART in limiting disease

progression and reversing or preventing immunodeficiency it has no effect on cells already infected with HIV and since it does not affect the latent reservoir it is not a cure. Individuals therefore require lifelong therapy which comes with challenges such as treatment adherence, drug resistance and the large economic burden on health sectors in resource-limited settings. Studies focusing on understanding HIV-1 persistence remains a priority research area in attempts to find a HIV cure. There are different definitions of cure: whereas the aim of an eradication cure is to rid the body of these long-lived HIV reservoirs, a functional cure aims to prevent or control viral rebound from reservoir cells in the absence of continued antiretroviral therapy. Considering the difficulty of eliminating the latent reservoir, scalable functional cures may be more achievable in the near future.

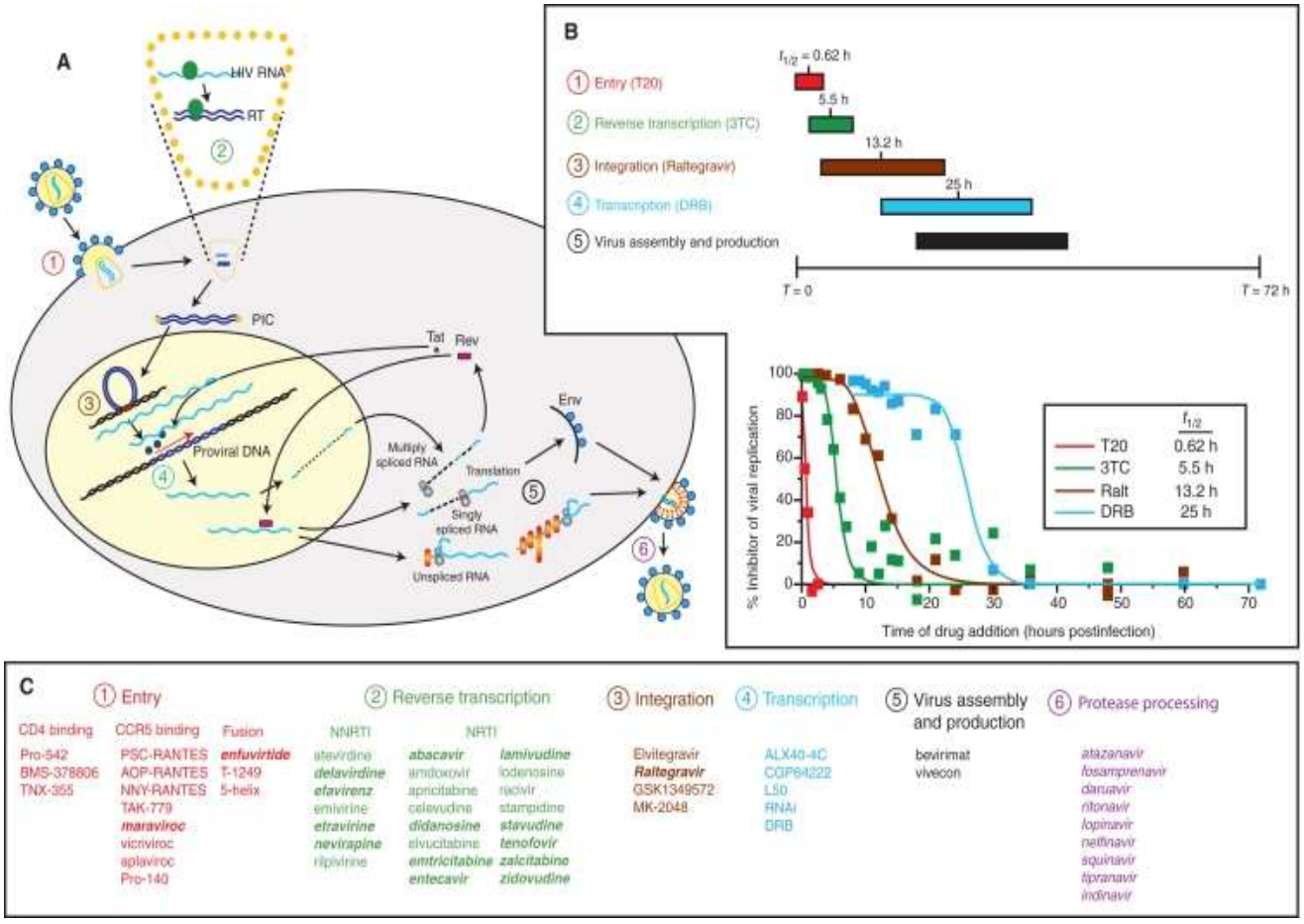


Figure 1.4 HIV-1 replication cycle and corresponding antiretroviral drug targets. (Source: Arts & Hazuda, 2012)

1.4.3 Markers of persistence and assays used to study HIV-1 persistence

HIV-1 persistence can be determined through the measurement of different biological markers using culture- or PCR-based assays. The true latent reservoir contains replication-competent virus, but other forms of HIV-1 DNA and cell-associated RNA have been shown to contribute to HIV

pathogenesis and HIV persistence through the formation of viral proteins that can induce immune activation or inflammation (Avettand-Fènoël *et al.*, 2016; Rouzioux and Avettand-Fènoël, 2018).

Measuring replication-competent virus using a viral outgrowth assay (VOA) remains the gold standard approach to quantify the true latent reservoir. Resting CD4+ T cells are isolated and purified from the patient. The cells are plated at limiting dilutions and phytohemagglutinin (PHA) is added along with irradiated peripheral blood mononuclear cells (PBMCs) to induce global T cell activation which leads to a reversal of latency and the release of virus (Eriksson *et al. 2013*; Finzi *et al.,* 1997). A p24 antigen detection assay or a reverse transcription-PCR assay are usually used to measure the antigen or virus released into the cell culture supernatant after one round of stimulation (Bruner *et al.,* 2015). It is important to note that after one round of stimulation only a subset of cells are activated. Latency reversal is stochastic and some studies have shown that upon another round of stimulation more virus could be recovered (Ho *et al.,* 2013). Therefore, VOA underestimates the size of the latent reservoir and may be referred to as the minimal estimate of the frequency of latently infected cells. Furthermore, the assay requires a large blood volume, it is expensive and labour intensive. However, despite the arduous nature of the assay and the underestimation of latent reservoir size, VOA only measures replication-competent virus and excludes defective and non-integrated forms of HIV-1 DNA therefore providing the best depiction of the true latent reservoir persisting in individuals on suppressive ART.

PCR-based assays offer high-throughput analysis and they are time and cost-efficient. Many of these assays are highly sensitive and require relatively small sample volumes which are suitable criteria for paediatric studies. Most PCR-based assays cannot distinguish between replication-competent virus and defective or non-integrated forms of HIV-1 DNA, but despite this limitation these assays provide valuable information about HIV pathogenesis and the overall proviral landscape which contain HIV-1 DNA forms that may produce viral proteins that may contribute to HIV persistence or the maintenance of cell populations containing replication-competent virus. Total HIV-1 DNA is measured using a real-time PCR assay (qPCR) or a droplet digital PCR (ddPCR) assay which targets a conserved region in the HIV-1 genome (either HIV-1 *gag, pol* or the LTR region) (Sharaf & Li, 2017). Both assays involve the isolation of PBMCs or CD4+T cells followed by extraction of nucleic acid, however, the detection or quantification of total HIV-1 DNA differs between these assays. Real-time PCR monitors the reaction in real-time and uses fluorescent probes for detection and quantifies total HIV-1 DNA using a standard curve. Droplet-digital PCR measures endpoint fluorescence and provides an absolute quantification through the use of Poisson distribution statistics. As mentioned, these assays cannot distinguish between replication-competent virus and defective or non-integrated forms of HIV-1 DNA. An assay that addresses this issue is the *Alu-gag* PCR assay which measures integrated proviral HIV-1 DNA. It targets Alu elements which are present in the human genome in high copy numbers and also targets an area in the HIV-1 *gag* gene, (Bruner *et al.,* 2015; Sharaf and Li, 2017). This assay more

accurately quantifies HIV-1 integrated DNA if integration occurred in a close proximity to Alu sequences in the human genome and a correction factor is used to quantify proviruses integrated far from Alu sequences. Despite this limitation, the measurement of integrated HIV-1 DNA is the best measurement that correlates with the VOA measurement (Kiselinova *et al.*, 2016).

In summary, total HIV-1 DNA is a valuable biomarker of the HIV-1 reservoir because it provides an overview of all infected cells that may contribute to HIV persistence in patients who are on suppressive ART. It may also be predictive of the size of the replication-competent reservoir size in ART suppressed patients (Kiselinova *et al.*, 2016). Table 1.1 summarises a few assays used to monitor HIV-1 persistence.

Table 1.1 Assays used to measure HIV-1 persistence

Assay	Measurement	Advantages	Disadvantages
Viral outgrowth assay (VOA)	Measures replication competent virus released from resting CD4+ T cells using reverse transcription PCR (RT-PCR) or p24 antigen ELISA.	Quantifies replication-competent virus excluding defective proviruses from the measurement	Underestimates latent reservoir size Labour intensive and time consuming Expensive assay Large sample volume which is not always feasible especially in paediatric cohorts
Real-time PCR (qPCR)	Total HIV-1 DNA including defective proviruses and unintegrated HIV-1 DNA	The latest assays are highly sensitive	It does not distinguish between replication competent virus and defective or unintegrated virus Different qPCR assays target different conserved regions and measure HIV-1 DNA relative to a standard. Therefore the measurement may not always be comparable to one another.

Droplet digital PCR (ddPCR)	Total HIV-1 DNA including defective proviruses and unintegrated HIV-1 DNA	Provides absolute quantification and is a more precise measurement in comparison to qPCR	It does not distinguish between replication competent virus and defective or unintegrated virus
***Alu-gag* PCR**	Integrated proviral HIV-1 DNA	Integrated DNA is a more comparable measurement to VOA	Limited to detection of proviruses close to the Alu sequences in the human genome Detects defective proviruses

1.5 HIV-1 Cure strategies

As mentioned above, the establishment of HIV-1 latent infection in the form of reservoirs remains the largest obstacle to achieving a cure. There are two main strategic aims driving HIV-1 cure research, namely achieving HIV-1 remission (also known as a functional cure) or achieving complete HIV-1 eradication. Regardless of the aim, the effect of latent reservoirs on both strategic efforts differ, but remains a challenge to address to attain the goal of a cure.

1.5.1 HIV-1 eradication

There are currently only two cases where HIV eradication succeeded: the so-called “Berlin patient”, Timothy Ray Brown (Hütter *et al.*, 2009), and the recent London patient (Gupta *et al.,* 2019), both had to undergo haematopoietic stem cell transplants for leukaemia and Hodgkin’s lymphoma, respectively. Myeloablative therapy (radiation and chemotherapy in case of the Berlin patient and chemotherapy alone in the case of the London patient) and graft versus host disease probably both contributed to reducing the number of infected cells. However, probably most importantly, their clinicians were able to find a matching donor who had the CCR5 delta-32 deletion, which provided new blood cells with resistance to HIV. Haematopoietic stem cell transplants without providing resistant cells were associated with delayed rebound but no cure and attempts to repeat this curative approach with CCR5 delta-32 deletion of autologous cells had not yet been able to reproduce a cure, although some preclinical data are promising (Henrich *et al. ,*2014).

The most investigated approach to reduce HIV reservoirs is the “shock and kill” strategy. In principle it involves the use of latency reversing agents (LRAs) to stimulate latent infected cells to release virions after which the immune system is expected to clear virus infected cells through immune-mediated cytolysis or apoptosis (Deeks *et al.*, 2016). This is done while the patient is on

therapy to prevent new rounds of replication of the virus released from the reservoir. Furthermore, in addition to the reversal of latency the immune system must be induced to kill cells infected with HIV once they are reactivated, specifically the recognition of infected cells by HIV-1 specific cytolytic T lymphocytes (CTLs). However, despite some evidence of latency reversal in a small proportion of infected cells, no studies have shown reservoir reduction. There are challenges that may prevent CTLs from successfully eliminating reactivated infected cells namely inherent resistance to the killing of cells harbouring replication competent HIV (Huang *et al.,* 2018), escape mutations in dominant CTL epitopes, LRAs may inhibit CTL function, some reservoirs occur in tissues that CTLs are not able to access and exhaustion of HIV-1 specific CTLs which make them ineffective in eliminating infected cells (Sengupta & Siliciano, 2018). Achieving complete eradication is a daunting task that requires assays and technology that might not be readily available and currently the more feasible strategy towards achieving a cure is achieving HIV-1 remission.

1.5.2 HIV-1 remission

This strategy can be defined as follows: the patient maintains a plasma viral load below limit of detection (suppressed) as a result of good immune control, retains normal immune function and remains non-infectious, in the absence of ART. This is also commonly referred to as a functional cure. There are various studies that have shown promising outcomes towards achieving remission, however, in some cases patients have experienced viral rebound after treatment cessation. One such study is the case of the Mississippi child who initiated ART within 30 hours of birth for a continuous period of 18 months and then discontinued therapy while the viral load remained undetectable for a period of more than two years (Persaud *et al.*, 2013). Subsequently, viral rebound occurred in the absence of HIV-specific immune responses and viraemia reached pre-therapy levels (Luzuriaga *et al.*, 2015). Studies including an analytical treatment interruption period provide valuable information about virological and immunological factors during the absence of ART. In the Viro-Immunologic Sustained Control after Treatment Interruption (VISCONTI) cohort 14 adults who initiated therapy within primary HIV infection (PHI) for a period 36.5 months all maintained a viral load of less than 400 copies per millilitre for a median of 7.4 years after interruption (Sáez-Cirión *et al.,* 2013) these individuals exhibited post-treatment immune control. A recent case study of a South African child who received ART at age 61 days and was interrupted at 50 weeks of age maintained post-treatment immune control for a period of 9.5 years with undetectable plasma HIV-1 RNA and HIV-1 DNA (Violari *et al.,* 2019). The child presented an immune profile similar to uninfected children that included a high CD4:CD8 ratio, low T cell activation and low CCR5 expression.

1.6 Early diagnosis and early ART initiation

1.6.1 Impact of early diagnosis

Early diagnosis of HIV-1 infection enables early initiation of therapy therefore preventing on-going replication and the infection of new cells thereby limiting the size of the reservoir that is seeded. Furthermore, because of the vulnerability of the infant immune system very early infant diagnosis (VEID) and subsequent early therapy initiation leads to a decrease in infant morbidity and mortality (Lilian *et al.,* 2013). The Children with HIV early antiretroviral therapy (CHER) randomised trial study has shown that initiating ART within 6 to 12 weeks of age can reduce infant mortality by 76% and decrease clinical disease progression by 75% (Violari *et al.,* 2008). VEID may also be seen as way to monitor perinatal mother-to-child-transmission rates to ensure that PMTCT programs are effective. A recent study has shown that the identification of HIV-1 infection at a later stage (median 6 months of age) was associated with higher risk of mortality in infants when compared to diagnosis as part of a PMTCT follow-up program (median age 1.6 months of age) (Abrams *et al.,* 2017). However, it is also important to consider that although rapid diagnosis for infants is recommended and available in many clinical settings, follow-up and retention in programs remains a challenge for health practitioners. In a recent short report two infants were diagnosed and initiated on a triple regimen ART within four hours of life. Despite this rapid response, both patients had frequent ARV dose adjustments, treatment adherence issues and eventually one was lost to follow-up (Clarke *et al.,* 2017). For early (or very early) infant diagnosis and early ART programs to succeed it requires a considerable effort from HIV clinicians and full support from parents or guardians.

1.6.2 Effects of early ART initiation on HIV-1 Persistence

Early therapy initiation following early diagnosis plays an important role towards achieving a cure for HIV. Early therapy limits the establishment of the latent HIV-1 reservoir and helps to sustain immune function which may be essential for immune mediated control, as required for a functional cure. Early studies has shown that early ART initiation results in low levels of markers of HIV persistence and undetectable HIV specific immune responses in children who initiated therapy before six months of age. At a median age of 6.3 years 60% of children had a low frequency of cells harbouring HIV DNA and none had detectable 2-LTR circles (a marker of residual viral replication). They also observed high frequencies of CD4+ T cells and low levels of cells expressing activation markers (Ananworanich *et al.,* 2014). Another study has shown that infants treated before six weeks of age who achieve viral suppression within six months have lower frequencies of cells containing replication-competent virus (Persaud *et al.,* 2012). Other evidence from adult cohorts has shown that treatment initiated during acute HIV infection (AHI) is important in limiting the reservoir size in comparison to adults treated at a later stage and after 12 months of

ART no replication-competent virus could be detected in patients who initiated therapy before or six months after seroconversion (Strain *et al.,* 2005). A recent cross-sectional study in children reported lower levels of HIV-1 DNA in children starting two months of age than those who started later (Kuhn *et al.*, 2018). Early therapy also affects the reservoir composition of infected CD4+ T cells subsets. Long-term non-progressors have a low infection rate of central memory CD4+ T cells and a larger number of anti-*gag* CD8+ T cells (Ananworanich *et al.,* 2015). Similar to what was observed in the VISCONTI cohort of post-treatment controllers, central memory CD4+ T cells contributed minimally to reservoir composition (Sáez-Cirión *et al.*, 2013). Other studies in adults (Martin *et al.,* 2017) and a case study of one child (Violari *et al.*, 2019) have also shown early therapy increases the probability of the post-treatment control. Another study aimed to investigate the effects of early ART on immune reconstitution in infants and found early treatment limited CD4 count decline, but did not restore it to levels seen in HIV uninfected infants (Lewis *et al.,* 2017).

Low HIV-1 DNA levels and smaller latent reservoirs increase the probability of achieving and sustaining HIV remission after treatment discontinuation by prolonging viral rebound (Hill *et al.,* 2014). The Mississippi child initiated therapy within 30 hours of birth for a period of 18 months and after discontinuing therapy had a undetectable viral load for two years before viral rebound (Luzuriaga *et al.*, 2015) and in an observational study an adult treated with prophylactic ART within 10 days of infection experienced viral rebound after 7.4 months following analytical treatment interruption (Henrich *et al.,* 2017). However, based on these studies very early ART initiation alone does not lead to ART-free remission, but it may delay viral rebound; and by reducing viral diversity and sustaining normal immune function, it may provide a good basis towards achieving a functional cure.

1.7 HIV-1 Decay Dynamics

The rate of decay of HIV-1 infected cells has not been extensively studied in children from resource limited settings and the factors that influence the rate of decay have not been fully elucidated. From reported studies the influence of the timing of ART initiation plays a significant role in HIV-1 DNA and HIV-1 RNA decay dynamics. One study investigated the effects of initiating therapy within the primary HIV infection (PHI) phase in comparison to initiation of therapy during chronic HIV infection (CHI). The initial decay rate during the first two years of combined ART was similar in the two groups, a half-life of 113 days (PHI) and 146 days (CHI). However, in the second phase the half-life of HIV-1 DNA was significantly shorter in the group of individuals treated during primary HIV infection, half-life of 25 years in comparison to a half-life of 377 years seen in those initiated during CHI. After viral suppression for a median of four years, HIV-1 DNA levels were lower in the earlier treated group (Hocqueloux *et al.,* 2013). Despite the similarity in the initial decay rate observed in these two groups, these findings suggest that starting ART early plays a role in restricting long term survival of infected cells that could inhibit HIV remission strategies.

Other studies in adults have also shown that initiation of therapy during acute HIV infection results in a much faster initial decline in HIV-1 DNA levels and HIV-1 DNA set point is rapidly reached (Ananworanich *et al.,* 2016; Laanani *et al.,* 2015).

In children a similar trend is seen, but the decay rate observed is much faster in comparison to adults. In children initiating ART within 2.6 months of age the HIV-1 DNA concentration decayed to as low as 1.0 log copies per million cells after two years (Luzuriaga *et al.,* 2014) and in children who initiated around a median of two months of age the median half-life was 53 days (Uprety *et al.,* 2015) and in children who initiated ART before three months of age the half-life was 107 days (McManus *et al.,* 2016). These findings further support the role of initiating ART as soon as possible following infection to ensure rapid decay of HIV-1 infected cells. Thereby limiting the size of the latent reservoir that may be established.

1.8 Rationale

1.8.1 Summary of Literature review

The global effort to increase access to antiretroviral therapy has reduced mortality rates among HIV-1 infected individuals, it has prevented more transmission events and has increased life expectancy among HIV positive individuals. However, the sustainability of life-long ART is threatened by the cost, side effects of long term usage and the potential accumulation of drug resistance mutations. Research continues towards finding a functional cure that will allow patients to discontinue therapy and remain virologically suppressed and maintain good immune control without ART. The major barriers to achieving this is the early establishment of a persistent latent reservoir of infected memory CD4+ T cells and the inability of most patient immune responses to control replication and prevent immune function decline. In the majority of patients, upon treatment cessation HIV-1 reservoirs stochastically reactivate from a latent state and virus replication continues.

The impact of very early treatment on the long-term survival of infected cells requires further investigation. The majority of reported studies have focused on adult cohorts and there remains a gap to provide information related to paediatric cohorts especially from resource limited settings. Furthermore, there are few reports providing longitudinal data for HIV-1 DNA and HIV RNA kinetics in children and associating clinical factors influencing the decay of infected cells.

1.8.2 Research questions

Our study aims to address the following research questions.

i. Is there a significant difference in the decay rate seen in infants treated within days after birth versus children who are treated weeks or months later?
ii. What factors influence HIV-1 DNA and HIV-1 RNA decay in children?

1.8.3 Aims and Objectives

This is a descriptive longitudinal study that aims to describe HIV-1 DNA and HIV-1 RNA decay in children and determine what factors could be influencing decay. Figure 1.5 gives an overview of the aims and objectives that have been set for this research project to answer the above mentioned research questions.

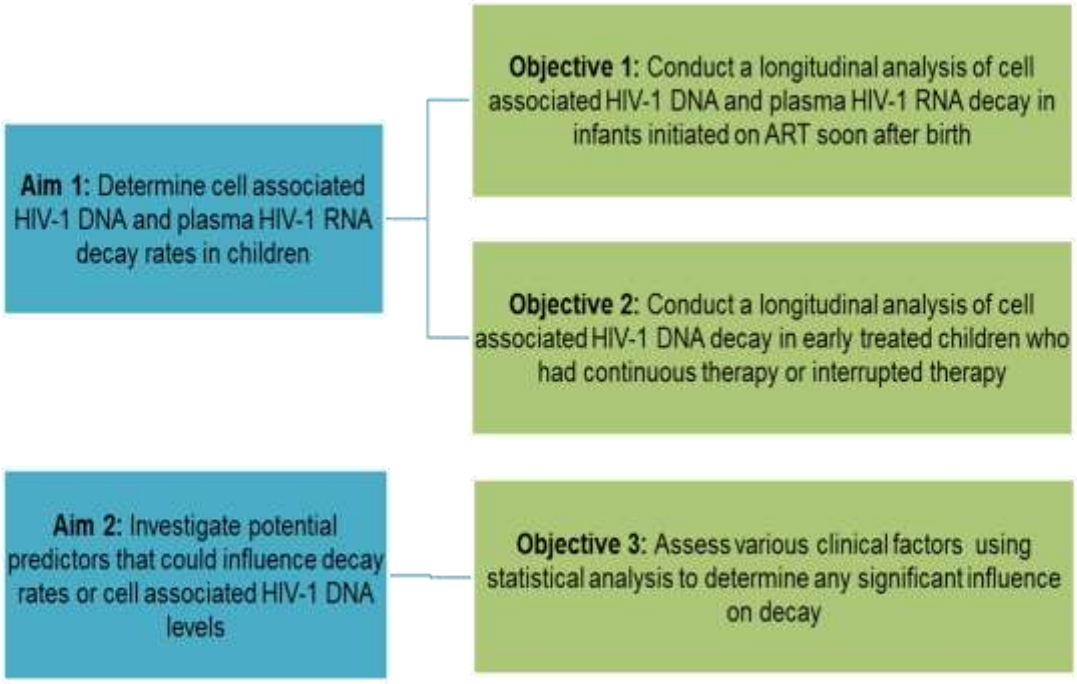


Figure 1.5 Overview of the aims and objectives for this research project.

Chapter 2: Materials and Methods

2.1 Ethical considerations

This research project falls under a larger study which was granted ethical approval by the Health Research Ethics Committee of Stellenbosch University, South Africa. Approval was granted on the 20 September 2017 with the following ethics reference number, M14/07/029.

2.2 Study specific definitions

2.2.1 Total HIV-1 DNA and plasma HIV-1 RNA

In this study total HIV-1 DNA refers to the total HIV-1 cell associated DNA in peripheral blood mononuclear cells that have been extracted from whole blood. Total HIV-1 DNA was measured using a real-time PCR assay targeting the integrase gene. Plasma HIV-1 RNA refers to the free plasma HIV-1 RNA load measured in the plasma using commercial diagnostic assays.

2.2.2 Total HIV-1 DNA decay

HIV-1 DNA decay refers to the change in total HIV-1 DNA (an estimate of the total number of infected cells) over time (Josefsson *et al.,* 2011). To perform linear regression total HIV-1 DNA was log transformed to study the decay of HIV infected cells over time.

2.2.3 Predictor

For the purpose of this study predictors are clinical or laboratory measurements that may explain or predict differences in HIV-1 DNA levels or rate of change of HIV-1 DNA when included in a statistical model. If predictors reach statistical significance and independently predict an outcome in a multivariate model it does not imply that they are the cause of the outcome, but merely that the association is statistically significant.

2.2.4 Continued treatment

Refers to the phase of therapy since treatment initiation in therapy uninterrupted patients or since therapy re-initiation in therapy interrupted patients.

2.3 Study design and participants

This research project aimed to investigate total HIV-1 DNA kinetics and plasma HIV-1 RNA kinetics in two different cohorts. Predictors associated with decay kinetics were only studied in one of the cohorts due to readily available clinical data. Below is a schematic giving an overview of the steps employed in this project to achieve the aims and objectives of this study.

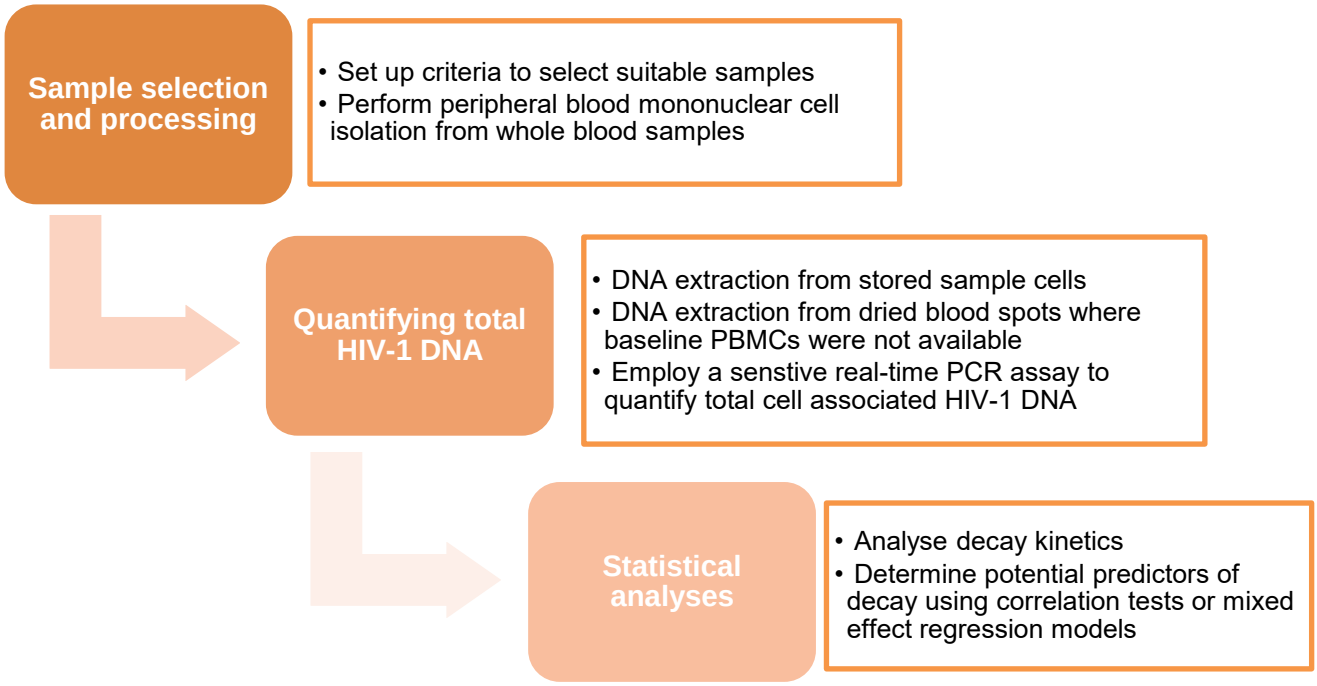


Figure 2.1 Schematic of overall study design

Cohorts studied and participant selection criteria

2.3.1 Very early infant diagnosis (VEID) cohort:

Infants were diagnosed as part of a public health sector birth HIV-1 diagnosis program in Cape Town, South Africa. To confirm HIV-1 infection at least two positive HIV-1 nucleic acid test results on two separate samples were needed. A quantitative, COBAS® AmpliPrep/COBAS® TaqMan® (CAP/CTM) HIV-1 version 2.0 or HIV-1 Qualitative version 2.0 (CAP/CTM) (Roche Molecular Diagnostics, Pleasanton, CA) tests were used. Following confirmation of test results, children were enrolled into an HIV-1 reservoir and neurodevelopment study. For the purpose of this Master's project a subset of 11 were studied (inclusion criteria below). All children were part of a Prevention of mother-to-child transmission (PMTCT) program that consisted mainly of a dual regimen of nevaripine (NVP) and zidovudine (AZT), replaced by triple combination therapy once diagnosed as HIV positive, whereas some children diagnosed at birth were immediately initiated on triple combination therapy (consisting of AZT, 3TC (lamivudine) and NVP) as described in Table 2.1. Nevaripine was replaced by lopinavir-ritonavir (LPV/r) at corrected age of 42 weeks and AZT replaced by ABC (abacavir) at approximately 3 months of age. Clinical visits and viral loads tests were done every 3 months. Plasma HIV-1 RNA was quantified with the Roche (CAP/CTM) version 2.0, with a 100 copies per millilitre limit of detection for a 200 microliter plasma input.

2.3.2 Children with HIV early antiretroviral (CHER) randomised trial cohort and Post-CHER cohort

The CHER study was a clinical trial conducted in two locations in South Africa; in Johannesburg at the HIV Research Unit, Chris Hani Baragwanath and in Cape Town at the Children's Infectious

Diseases Clinical Research Unit, Tygerberg Hospital (Cotton *et al.*, 2013; Violari *et al.*, 2008). Infants were recruited into the study following confirmation of HIV infection by a positive PCR test for HIV-1 DNA. Other entry criteria were a plasma HIV-1 RNA PCR test result of greater than 1000 copies per millilitre. Children with a CD4 percentage of 25% or more formed part of the main trial (Part A) whereas children with a CD4 percentage below 25% were assigned as Part B (and were excluded from the primary analysis which compared immediate to deferred therapy). Following recruitment into the study participants from Part A were randomised into three arms defined by three different treatment strategies and Part B participants were only randomised into two of the three arms (excluding the deferred arm as they required immediate therapy). The arms are as follows:

- Arm 1: Deferred ART therapy. Children were not started immediately on therapy.
- Arm 2: Early time limited therapy for 40 weeks. Children received therapy for 40 weeks and subsequently therapy was interrupted.
- Arm 3: Early time limited therapy for 96 weeks. Children received therapy for 96 weeks and subsequently therapy was interrupted.

Within each arm the following immunological criteria were used either to initiate therapy in Arm 1 (delayed therapy arm) or re-initiate therapy in Arm 2 or Arm 3: A CD4 percentage of less than 20% or in the case of infants younger than 12 months a CD4 percentage less than 25% or a CD4 count of less than 1000 cells per cubic millimetre. Participants were started on a first line ART regimen that consisted of zidovudine (AZT) and lamivudine (3TC) with lopinavir-ritonavir (LPV/r). The second line regimen consisted of didanosine (DDI), abacavir (ABC) and, and nevaripine (NVP) or efavirenz (EFV). Following the conclusion of the clinical trial a subset of children were retained in post-CHER descriptive studies to investigate neurocognitive outcomes and HIV-1 reservoirs. The participants for this Master's project were selected from the Cape Town site, who were retained in post-CHER follow up studies. Clinical assessments (such as monitoring CD4 counts) and viral load tests were done at regular study visits. We selected participants based on specific criteria listed below and Table 2.2 summarises the patients included in this project. .

2.3.3 Participant inclusion criteria

To answer the research questions set out in this study the following criteria were set to select participants from the above mentioned cohorts.

I. VEID cohort

   Participants were selected if:

   - The individual initiated ART within eight days of birth.
   - The individual had a detectable total HIV-1 DNA baseline sample available.
   - There were two stored PBMC samples available while on treatment.

II. CHER and Post-CHER cohort

Participants were selected if:

- The individual had a baseline sample before therapy initiation if the patient was on continuous therapy (CHER Arm 1).
- The individual had a baseline sample before therapy re-initiation if the patient was subjected to therapy interruption (CHER Arm 2 or Arm 3).
- Participants had two early time points and at least 1 late time point (Post-CHER sample).

Table 2.1 Participants selected from the VEID cohort. "S" indicates virologic suppressed infants and "V" viraemic infants. Virologic suppression was defined as a continuous downward trend in plasma HIV-1 RNA and a HIV-1 RNA load <100 copies/mL after 6 months on ART. Participants not meeting these criteria were classified as viraemic.

Patient ID	Gender	Child PMTCT	Child PMTCT Drugs	Age(days) at ARV Start #	Age (days) recruited*
S2	Male	Yes	Immediate cART	0	35
S3	Female	Yes	NVP, AZT	3	18
S4	Female	Yes	AZT/3TC	5#	7
S5	Male	Yes	NVP, AZT	7	22
S6	Female	Yes	Immediate cART	0	30
S7	Female	Yes	NVP, AZT	6	12
S9	Male	Yes	NVP, AZT	8	8
V1	Female	Yes	NVP	6	22
V3	Male	Yes	NVP, AZT	8	54
V4	Male	Yes	NVP, AZT	7	20
V5	Female	Yes	NVP, AZT	4	10

A 2-5 day delay between initial dual therapy and triple therapy. The initial combination antiretroviral regimen in all was azidothymidine (AZT), lamivudine (3TC) and nevirapine (NVP). NVP was replaced by lopinavir/ritonavir (LPV/r) at a corrected age of 42 weeks; AZT was replaced by abacavir (ABC) at approximately 3 months of age. *On the recruitment day the first PBMC samples to investigate HIV-1 reservoirs were collected.

Table 2.2 Participants selected from the CHER/Post-CHER cohort.

CHER Treatment Strategy	Patient ID	Gender	Age at initiation (days)	Initial ART regimen
Part A Arm 1	330812	Female	256	AZT, 3TC, LPV/r
	335366	Female	135	AZT, 3TC, LPV/r
	335726	Female	139	AZT, 3TC, LPV/r
	336926	Male	290	AZT, 3TC, LPV/r
	339606	Male	259	AZT, 3TC, LPV/r
	338956	Female	174	AZT, 3TC, LPV/r
	330446	Female	450	AZT, 3TC, LPV/r
	331976	Female	129	AZT, 3TC, LPV/r
Part A Arm 2	339316	Male	51	AZT, 3TC, LPV/r
	333676	Female	51	AZT, 3TC, LPV
	336306	Female	51	AZT, 3TC, LPV/r
	337756	Female	70	AZT, 3TC, LPV/r
	338346	Female	49	AZT, 3TC, LPV/r
	338796	Female	64	AZT,3TC, LPV/r
	339046	Male	51	AZT, 3TC, LPV/r
	339486	Male	52	AZT, 3TC, LPV/r
	339736	Female	65	---
	340206	Female	50	AZT, 3TC, LPV/r
	341622	Male	64	AZT, 3TC, LPV/r
	145046	Male	41	AZT, 3TC, LPV/r
	330636	Male	52	AZT, 3TC, LPV/r
	335496	Female	58	AZT, 3TC, LPV/r
	331232	Female	84	AZT, 3TC, LPV/r
	331766	Female	54	AZT, 3TC, LPV/r
	332196	Male	65	AZT, 3TC, LPV/r
	333946	Male	55	AZT, 3TC, LPV/r
Part A Arm 3	340566	Male	60	AZT, 3TC, LPV/r
	339156	Male	53	AZT, 3TC, LPV/r
	333716	Female	70	AZT, 3TC, LPV/r
	336456	Female	64	---
	330556	Female	49	AZT, 3TC, LPV/r
	341286	Male	55	AZT, 3TC, LPV/r
	331122	Male	82	---
	331596	Female	46	AZT, 3TC, LPV/r
	333226	Female	68	AZT, 3TC, LPV/r
	338686	Male	54	AZT, 3TC, LPV/r
Part B Arm 2	360672	Male	68	AZT, 3TC, LPV/r
	360132	Female	50	AZT, 3TC, LPV/r
	360392	Male	79	AZT, 3TC, LPV/r
	360712	Male	84	AZT, 3TC, LPV/r

2.4 Peripheral blood mononuclear cell (PBMC) isolation

This protocol was performed in a Biosafety level 2 laboratory. Whole blood (EDTA) samples were received from participants and transferred to a Falcon tube (either 15 mL or 50 mL depending on the sample volume). The samples were then centrifuged (Rotanta 460 R, Hettich, USA) at 967 x g for ten minutes with the brake and acceleration step set to the highest level on the centrifuge. This step separates plasma from other whole blood components such as white blood cells, red blood cells and platelets. Following centrifugation the plasma supernatant was removed and stored at -80^{0}C for other assays. The remaining cells were re-suspended with an equal amount of 1X phosphate-buffered saline (PBS) (Lonza, Switzerland) in relation to the amount of plasma removed. The reconstituted blood was layered onto a density gradient cell separation medium called Histopaque Ficoll (Sigma-Aldrich, USA). For large blood volumes approximately 15 mL of media was used and for smaller blood volumes between 5-6 mL of Ficoll was used.

After layering the blood on the separation media the samples were centrifuged for 30 minutes at 434 x g (with the brake and acceleration step removed or set to the lowest level). After the centrifugation step the presence of four layers was evident and the buffy white coat containing the PBMCs was removed by extending a bulb pipette through the above layer and carefully extracting the cells. The cells of each sample were transferred to an empty Falcon tube (15 mL or 50 mL depending on initial blood volume size) and 1X PBS was added to fill up each tube to maximum capacity. The cells were centrifuged at 278 x g for 17 minutes (with the brake and acceleration step removed or set to the lowest level). After this wash step the PBS was carefully decanted and the pellet was re-suspended in the excess PBS left in the tube. If the initial sample volume was above 20 mL, then the wash step was repeated by filling up the tube with 1X PBS and centrifuging the tubes again. For smaller initial blood volumes, 1-5 mL of 1X PBS was added to the reconstituted pellet after the first wash step in preparation for cell counting. After the second wash of the larger blood volume, the PBS was again decanted and the cell pellet re-suspended in the excess PBS. For cell counting, 20 mL of 1X PBS was added to the reconstituted pellet and Trypan Blue (Bio-Rad Laboratories, USA) was used to stain the cells to count the cells using a TC20™ Automated cell counter (Bio-Rad Laboratories, USA).

Fifty microlitres of Trypan Blue was mixed with 50 µL of cells and then 10 µL of this mixture was pipetted onto both sides of a counter slide (side A and side B). The outside of the slide was wiped with 70% ethanol and then placed into the cell counter. The lower gate was set to 6 µM and the upper gate set to 17 µM. A total of four counts were read (two on each of the slide) and the average cell count was used in further calculations to determine the amount of cryopreservation media needed (Addendum A1). The cells were then centrifuged at 275 x g for ten minutes and the media prepared using dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS).After centrifugation, the PBS was decanted and the pellet reconstituted in the remaining PBS and then

the cryopreservation media was added to the cells in a drop wise manner while swirling the tube to ensure that the cells were evenly mixed into the media. Then 1 mL aliquots of 2.5 million cells were stored in a Mr Frosty overnight at -80˚C and then transferred to liquid nitrogen for long term storage.

2.5 DNA extraction from peripheral blood mononuclear cells (PBMCs)

This protocol was performed in a Biosafety level 2 laboratory. Samples were removed from liquid nitrogen and placed at -80˚C overnight to equilibrate before carrying on with the extraction protocol. The following day and prior to removing samples from -80˚C the dry baths (AccuBlock™, Labnet International, USA) were set to 37˚C and 42˚C, respectively. Samples were then removed from -80˚C and placed on dry ice. During this time, the Roswell Park Memorial Institute (RPMI) media was heated at 37˚C for approximately ten minutes (1 mL of media per sample). Following this the samples were placed at 37˚C for approximately two minutes (one sample at a time). Samples thaw at different rates and must not be left unattended to ensure cells are not destroyed. After the samples were thawed, 1 mL of RPMI media was added drop-wise to each sample. Samples were stored at various cell concentrations, for the purpose of this study we only aimed to extract from 800 000 to 1 000 000 cells so that we extract a sufficient amount of DNA, but also not too much that it inhibits downstream assays (i.e. the real time PCR assay). Therefore, after adding RPMI media to each sample the cells were split into pre-calculated volumes to ensure that we only extracted from within the range of the cell concentrations mentioned above. The split sample volumes are transferred from the cryovials to 1.5 mL tubes and centrifuged (Prism R, Labnet International, USA) at 500 x g for five minutes. The supernatant was carefully removed and the cell pellets were stored on dry ice until all individual samples have undergone the above steps. The remaining protocol was carried on with one aliquot of cell pellet and the rest of the cell pellets were stored at -80^{0}C.

Thereafter, 100 µL of a Guanidinium Hydrochloride (GuHCl, 8 M, Sigma-Aldrich, USA) and Proteinase K (20 mg/mL, ABI Scientific, USA) solution is prepared for each sample by adding 50 µL of Proteinase K per 1 mL of GuHCl+/ProK required (Addendum A2). Once the solution was made a 100 µL was added to each cell pellet and immediately sonicated (Omni Sonic Ruptor 400, Omni International, USA) for 10 seconds. If the cell pellet had not completely dispersed it was further sonicated for another five seconds. Following this the samples were placed at 42^{0}C for one hour. During the incubation step 400 µL of Guanidinium Isothiocyanate (GuSCN, 6 M, Sigma-Aldrich, USA) and glycogen (Gly, 20 mg/mL, ABI Scientific, USA) solution was prepared for each sample by adding 30 µL of glycogen per milliter L of GuSCN+/Gly required (Addendum A2). After the incubation step samples were removed from the dry bath and 400µL of GuSCN+/Gly were added to each sample. Each sample was vortexed for 10 seconds and placed back on the dry bath for a further 10 minutes. Then 500 µL of 100% isopropanol (at room temperature) was added to

each sample and vortexed for 10 seconds at a high intensity and centrifuged for 10 minutes at 21 000 x g to pellet the nucleic acid.

The supernatant was then carefully removed and 750 µL of 70% ethanol (at room temperature) was added to each sample. The samples were then vortexed for 10 seconds and centrifuged for 10 minutes. After this wash step the supernatant was removed and the samples spun at 21 000 x g for 15 seconds. After this quick centrifugation step any residual ethanol was removed and the pellets were air dried by keeping the tubes open until the pellet appears semi-translucent. It took approximately 3-7 minutes to dry and had to be carefully monitored to prevent the pellets from over drying which may make it difficult to re-suspend the pellet. After the pellet had dried, 70 µL of 5 mM Tris-HCl (diluted from stock: 1 M Tris-HCl, pH 8.0, Sigma, USA) was added to each sample. The samples were sonicated for 10 seconds at 60% amplitude to properly dissolve the nucleic acid into solution. The extracted DNA was stored at -80^{0}C until needed for other assays.

2.6 Adapted method for DNA extraction from a dried blood spot (DBS)

2.6.1 DNA extraction from DBS

This protocol was performed in a Biosafety level 2 area. The same DNA extraction method in section 2.5 was adapted for the extraction of nucleic acids from dried blood spots. Figure 2.2 provides a brief overview of this method particularly highlighting the main amendments made to the existing method. Firstly, the amount of lysis solution used was increased and samples were stored overnight at 37^{0}C in this solution.

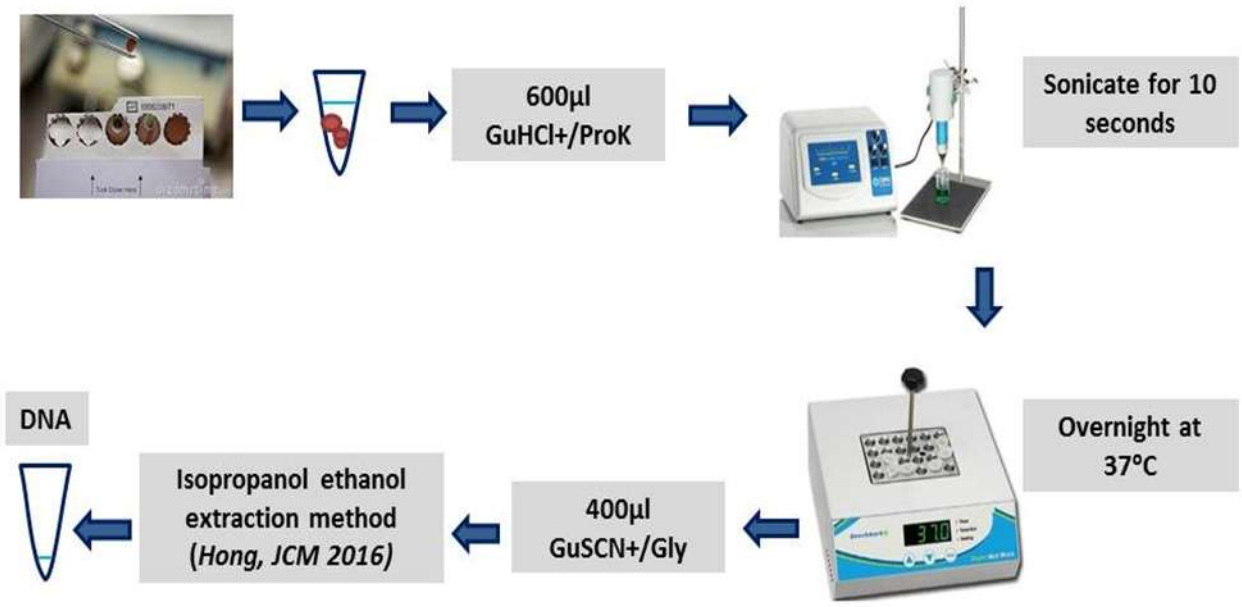


Figure 2.2 Overview of nucleic acid extraction from a dried blood spot (DBS).

For each sample one dried blood spot was punched out and placed in a 1.5 mL tube. A lysis solution consisting of Guanidinium hydrochloride and Proteinase K was prepared. Six hundred microlitres of the lysis solution was needed for each sample and the total volume was calculated according to formula in the addendum A2. The lysis solution was added to the sample and the sample was immediately sonicated for ten seconds at 60% amplitude. The samples were incubated overnight at 37^0C on a dry bath**.** The next day a Guanidinium isothiocyanate and glycogen solution was prepared (Addendum A2). The sample was taken off the heating block and in three new 1.5 mL tubes the total sample volume was split into three volumes consisting of 200 µL each. Four hundred microlitres of the GuSCN/+Gly solution was added to the samples and vortexed for 10 seconds. The samples were placed on the heating block for a further 10 minutes. Following this step 500µL of 100% isopropanol (stored at room temperature) was added to each sample and vortexed at a high intensity for 10 seconds. Samples were transferred to a centrifuge and spun at 21 000 x g for 10 minutes. The supernatant was removed and the pellet (consisting of the nucleic acid) was washed using 750 µL of 70% ethanol and vortexed for 10 seconds. Samples were stored overnight at -20˚C to properly wash the pellet and sufficiently dissolve any possible contaminants. The next day samples were spun at 21 000 x g for 10 minutes and the supernatant removed and then again spun for 15 seconds at 21 000 x g to remove any excess supernatant. The samples were dried on the bench top until the pellet was semi-translucent. Each dried pellet was re-suspended with 23.33 µL 5 mM Tris-HCl and stored overnight at -20˚C to allow the nucleic acid to properly dissolve into solution. Each sample that was initially split into three at the beginning of the protocol was pooled into one tube with a total volume of 70 µL and the DNA concentration and purity was determined using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, USA).

2.6.2 Determining the total amount of peripheral blood mononuclear cells in a dried blood spot

There were no baseline PBMC samples available for the VEID cohort therefore we sourced baseline dried blood spots as way to overcome this limitation. However, the DNA extraction protocol (described in Section 2.5) had to be amended to enable extraction of nucleic acids from a dried blood spot. In our standard protocol we extracted from PBMC samples which in comparison to DBS is a more pure sample containing mainly lymphocytes. DBS is a whole blood sample containing other cells such as granulocytes in addition to lymphocytes. To determine the amount of lymphocytes that were present in a dried blood spot the following experiment was set up. We obtained residual NHLS infant samples that tested HIV negative at approximately the same age as expected for positive baseline samples (between the ages of one to eight days old). A blood volume of at least 250 µL was needed to have enough blood to make a 50 µL dried blood spot and peripheral blood mononuclear cells were extracted from the remaining blood. The amount of samples available for selection was limited due to insufficient blood volumes below 250 µL and

other stored samples were haemolysed and not suitable for use in this experiment. Subsequently only two HIV negative samples were obtained, sample one (named Neg 1) had a blood volume of 250 µL and sample two (named Neg 2) had a blood volume of 300 µL. For each sample a dried blood spot was made on a Guthrie card and from the same sample PBMCs were isolated from the remaining blood according to the same method in section 2.4. DNA was extracted from the dried blood spot using the method in section 2.6.1 and DNA was extracted from the EDTA whole blood PBMC samples according to the method in section 2.5. A real time PCR assay was used to determine the amount of cells assayed by quantifying the total number of copies of CCR5 in each sample. The real-time PCR assay used is described in detail in section 2.7.2. Thereafter the total number of cells recovered from a dried blood spot (which would include polymorphonuclear cells) was expressed in relation to the number of peripheral blood mononuclear cells recovered from the same volume of processed whole blood.

2.7 Integrase cell associated total HIV-1 DNA assay (iCAD)

2.7.1 Preparation of integrase HIV-1 Subtype C DNA integrase standard for iCAD

A set of primers (Addendum A3) designed to amplify a 418 bp fragment from the HIV-1 subtype C integrase gene was used to generate amplicons from the pMJ4 plasmid (HIV-1 subtype C clone). The following master mix concentrations were used to prepare a master mix for 20 reactions (Table 2.3). We selected 20 reactions as a start to have enough product downstream especially after purification. The master mix was added to 20 PCR tubes and 5 µL of pMJ4 linearized plasmid was added to each tube. The reactions were run on a thermocycler (9700 PCR System, Applied Biosystems, USA) with the following cycle parameters listed below (Table 2.4). The products were run for 45 minutes at 90 volts on a 0.8% agarose gel alongside a 1 kb DNA ladder to determine if the reaction worked and the correct band size of 418 bp was observed. Nine microliters of loading dye and 45 µL of each product were mixed separately and loaded onto a gel containing EZ Vision® (VWR International. USA). The gel was viewed using the UVITEC Cambridge Gel Doc system.

Table 2.3 Master Mix calculations to generate 418 bp amplicon containing HIV-1 subtype C integrase insert

Reagent	Stock Concentration	Final Concentration	Volume per reaction (µL)
PCR grade H_2O	--	--	25.75
GoTaq Buffer	5X	1X	10
dNTPs	40 mM	1.25 mM	1
$MgCl_2$	25 mM	2 mM	4
P1	10 µM	0.4 µM	2
P2	10 µM	0.4 µM	2
GoTaq Enzyme	5 U/Ul	1.25 U	0.25

DNA	2 ng/µL	10 ng	5
Total	--	--	50

Table 2.4 Cycling parameters for endpoint PCR.

Step	Temperature	Time	
Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	
Annealing	44°C	30 s	30 cycles
Extension	72°C	30 s	
Final extension	72°C	7 min	
Hold	4°C	∞	

The 418 bp amplicon was gel extracted using the Qiaquick gel extraction kit (Qiagen, Netherlands) according to the manufacturer's instructions. Following gel extraction ethanol precipitation was performed to purify the sample from any salts carried over into the sample during extraction. The following steps were carried out: 3 M Sodium acetate equivalent to a tenth of the extracted DNA volume was added to the tube containing the DNA (for example if the extracted DNA volume is 200 µL then 20 µL of Sodium acetate was added). In addition to this, 100% ice cold ethanol was added to each tube. The amount of ethanol was equivalent to 3 times the volume of extracted DNA. The solution was thoroughly mixed using a vortex and then placed at -20^{0}C overnight to precipitate. The next day the tubes were centrifuged at 17000 x g for 30 minutes. The pellet was washed using 500 µL ice cold 80% ethanol and centrifuged for 10 minutes. The wash step was repeated and the ethanol was removed. The tubes were centrifuged at full speed for 10 seconds to remove any residual ethanol and the pellet air dried. Once dried the pellet was re-suspended in 200µL 5 mM Tris and thoroughly mixed using a vortex. The sample purity was determined using a NanoDrop 1000 and then 5 µL of product with 1 µL Novel juice (GeneDirex, USA) was run on a 1% gel with a 1 kb DNA ladder for 45 minutes at 90 V to ensure that the correct fragment size was gel extracted and purified. The DNA concentration was quantified using a Qubit® 2.0 fluorometer (Thermo Fisher Scientific, USA) with the Qubit® dsDNA HS Assay kit instructions provided by the manufacturer. The concentration, amplicon sequence and base pair length were used to calculate the exact DNA copy number using an Endemo DNA Copy number calculator available online (Addendum A4). After determining the DNA copy number ten-fold serial dilutions were performed. First the initial DNA copy number was diluted to 1 x10^{10} copies/µL and then ten-fold serial dilutions were performed down to a concentration of 1 x10^5 copies/µL. Serial dilutions were done using the following method: 900 µL of 5 mM Tris was dispensed into five 1.5 mL tubes and 100 µL of the 1 x10^{10} copies/µL (standard stock) was transferred into the first 1.5 mL tube labelled 1 x10^9. To mix the solution the tube was inverted 30 times and then incubated at 56^{0}C for five minutes. The tube

was then placed on wet ice for one minute and for each subsequent solution 100 µL of the solution was dispensed from the previous dilution into the next tube and diluted following the same method down to 1 x10^5 copies/µL. Figure 2.3 shows an overview of the above mentioned serial dilution. Single-use aliquots were made of the 1 x10^5 copies/µL HIV-1 subtype C integrase standard.

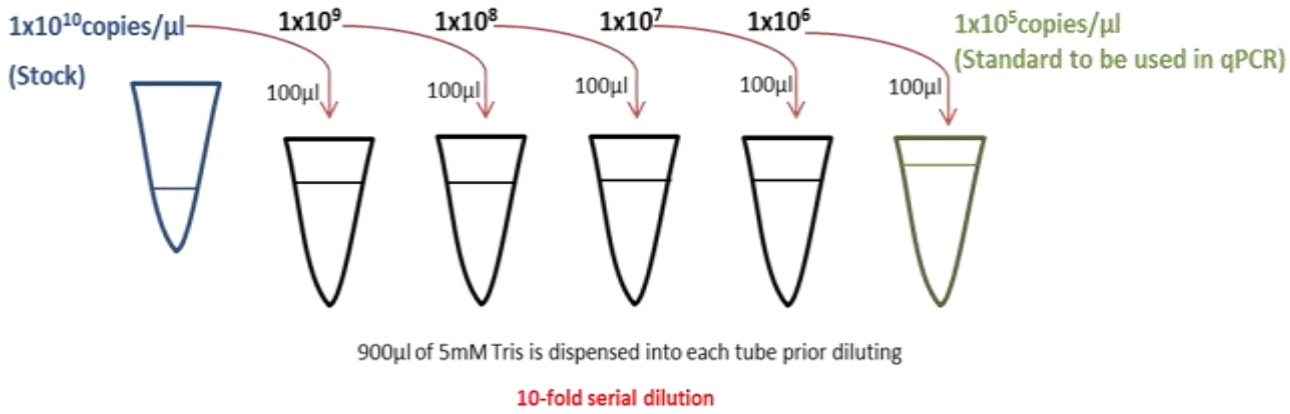


Figure 2.3 Basic overview of the method used to dilute HIV- 1 integrase standard

2.7.2 Limiting Dilution experiment to verify HIV-1 Integrase standard

A real time PCR limiting dilution assay was used to verify if the HIV-1 DNA integrase standard generated was at the correct concentration of 1 x10^5 copies/µL. A master mix for 96 reactions was prepared according to Table 2.5 (primers and probe are listed in the Addenda) and while on a cooling block, 15 µL of the master mix was dispensed into each well of a 96-well real time PCR plate (Bio-Rad Laboratories, USA). The plate was covered with an aluminium cover to prevent probe exposure to light and the standard which was prepared as in section 3.7.1 was diluted from 1 x10^5 copies/µL down to one copy per well (10 µL). Figure 2.4 shows the overall method used to perform the serial dilution, 108 µL of 5 mM Tris was dispensed into twelve tubes and 50 µL of the HIV-1 DNA integrase standard was transferred to the first tube labelled 3 x10^5 copies/10 µL and the pipette tip was changed before mixing 15 times by aspirating and dispensing using the micropipette. Subsequently to that, 50 µL of this new dilution was transferred to the next tube labelled 1 x10^5 copies/10µL and the process was repeated until 1 copy/10 µL was reached. The dilutions performed are a 1 to 3.2 dilution because 3.2 ≈ (is almost equal to) $10^{1/2}$ therefore every second dilution in the series is a ten-fold dilution. After completing the serial dilution, 10 µL of each dilution from 3000 copies/10 µL to one copy/10 µL were dispensed in replicates of 10 according to the plate layout in Figure 2.5. The plate was tightly sealed with clear plastic film and placed on a CFX Connect real-time PCR machine (Bio-Rad Laboratories, USA) and the following cycle conditions were used: Step 1: 95^{0}C for five minutes, Step 2: 95^{0}C for 15 seconds, Step 3: 60^{0}C for one minute and then step 2-3 is repeated for 50 cycles and then a final hold step at 37^{0}C was set.

After completion of the run the results were analysed using Bio-Rad CFX Manager version 3.0 software.

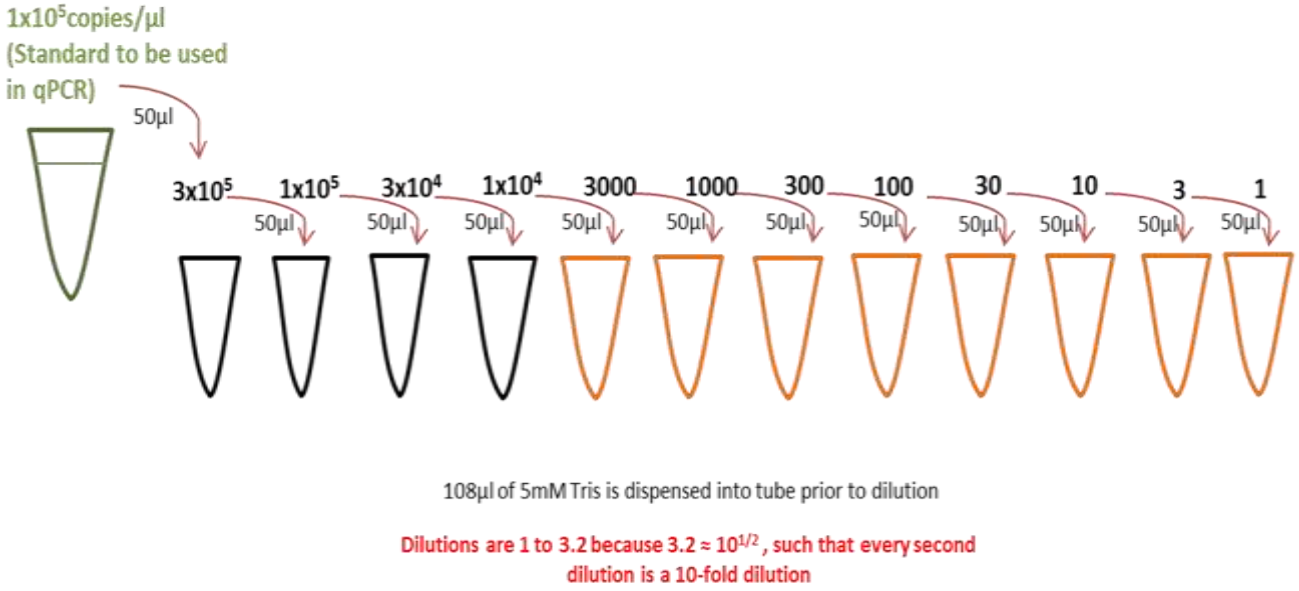


Figure 2.4 Basic overview of method used to perform dilution series.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 HIV	1 HIV	1 HIV	1 HIV	1 HIV	1 HIV	1 HIV	1 HIV	1 HIV	1 HIV	NTC	NTC
B	3 HIV	3 HIV	3 HIV	3 HIV	3 HIV	3 HIV	3 HIV	3 HIV	3 HIV	3 HIV	NTC	NTC
C	10 HIV	10 HIV	10 HIV	10 HIV	10 HIV	10 HIV	10 HIV	10 HIV	10 HIV	10 HIV	NTC	NTC
D	30 HIV	30 HIV	30 HIV	30 HIV	30 HIV	30 HIV	30 HIV	30 HIV	30 HIV	30 HIV	NTC	NTC
E	100 HIV	100 HIV	100 HIV	100 HIV	100 HIV	100 HIV	100 HIV	100 HIV	100 HIV	100 HIV	NTC	NTC
F	300 HIV	300 HIV	300 HIV	300 HIV	300 HIV	300 HIV	300 HIV	300 HIV	300 HIV	300 HIV	NTC	NTC
G	1000 HIV	1000 HIV	1000 HIV	1000 HIV	1000 HIV	1000 HIV	1000 HIV	1000 HIV	1000 HIV	1000 HIV	NTC	NTC
H	3000 HIV	3000 HIV	3000 HIV	3000 HIV	3000 HIV	3000 HIV	3000 HIV	3000 HIV	3000 HIV	3000 HIV	NTC	NTC

Figure 2.5 Plate layout for limiting dilution assay

2.7.3 Real-time PCR assay

An in-house real time PCR assay was used to quantify total HIV-1 DNA in peripheral blood mononuclear cells and dried blood spots. This assay was developed by our research collaborators and adapted to target the integrase gene in HIV-1 subtype C (Hong *et al.,* 2016) . This is an ultrasensitive assay with a limit of detection of 3 copies per million cells. It utilises two standards, an HIV-1 integrase DNA standard to quantify cell associated DNA and a CCR5 standard which is used to quantify the total amount of cells assayed in each sample as each diploid human cell (including peripheral blood mononuclear cells) contains two copies of this gene.

Prior to starting the assay the DNA concentration of each extracted sample was measured using a Nanodrop 1000 spectrophotometer. Sample concentrations above 120 ng/µL were diluted down to a final concentration of 120 ng/µL. This was to prevent over saturation of the assay with human genomic DNA which could lead to inhibition. Following this the appropriate amount of master mix was prepared according to the optimised reagent concentrations depending on the amount of samples to be assayed (Table 2.5).

Table 2.5 Master Mix calculations for real time PCR assay

HIV-1 and CCR5 Master mix (Sample DNA and Standard)*			
Reagent	**Stock Concentration**	**Final Concentration**	**Volume per reaction (µL)**
Molecular grade water	--	--	1.97
2X LCM	2X	1X	12.5
P1	100 µM	0.9 µM	0.23
P2	100 µM	0.9 µM	0,.23
Probe	100 µM	0.3 µM	0.075
Total cocktail	--	--	15
Sample	--	--	10
Total Volume			**25**

*Primers used can be found in Addendum A5

A 96 well plate was placed on a cooling block and 15 µL of the master mix distributed into the wells of the plate according to the layout in Figure 2.6. The plate was sealed with aluminium foil to prevent probe exposure to light. Invitrogen TaqMan Control genomic DNA was used as the CCR5 standard and serially diluted from a stock concentration of 16667 cells/µL down to 1042 cells/µL using a 1:1 dilution by combining 25 µL of stock DNA with 25 µL of 5 mM Tris solution and repeating until the desired concentration was reached. Before adding sample DNA to the wells containing CCR5 master mix the DNA extracted from participant samples were diluted to prevent inhibition of the real-time assay because CCR5 is abundant in genomic DNA. A 1:30 dilution of each sample was prepared by first preparing a 1:10 dilution by combining 10 µL of sample with 90

µL 5 mM Tris and then 10µL of the 1:10 dilution is combined with 20µL of 5 mM Tris to reach a dilution of 1:30 of each sample. Once all samples have been diluted the total HIV-1 DNA standard was prepared by serially diluting HIV-1 DNA from a stock concentration of 1 $x10^5$ copies/µL down to 3 copies per well. This was done by combining 18.75 µL of the standard HIV-1 DNA with 40.5 µL of 5 mM Tris and repeating this step until 3 copies per well was reached. After preparing all the necessary dilutions the samples and standards were aliquoted into the respective wells according Figure 2.6. The plate was covered with a clear film and the edges firmly secured to prevent evaporation during the PCR run. The plate was transferred on the cooling block to the CFX Connect (Bio-Rad Laboratories, USA) and the samples were run using the following cycle specifications: Step 1: 95^0C for five minutes, Step 2: 95^0C for 15 seconds, Step 3: 60^0C for one minute and then step 2-3 was repeated for 50 cycles and then a final hold step at 37^0C was set. After completion of the run the results were analysed using Bio-Rad CFX Manager version 3.0 software.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 HIV DNA	Sample 2 HIV DNA	Sample 3 HIV DNA	Sample 4 HIV DNA	Sample 5 HIV DNA	Sample 6 HIV DNA	Sample 7 HIV DNA	Sample 8 HIV DNA	Sample 9 HIV DNA	Sample 10 HIV DNA	Sample 11 HIV DNA	Sample 12 HIV DNA
B												
C												
D	Sample 1 CCR5 DNA	Sample 2 ICCR5 DNA	Sample 3 CCR5 DNA	Sample 4 CCR5 DNA	Sample 5 CCR5 DNA	Sample 6 CCR5 DNA	Sample 7 CCR5 DNA	Sample 8 CCR5 DNA	Sample 9 CCR5 DNA	Sample 10 CCR5 DNA	Sample 11 CCR5 DNA	Sample 12 CCR5 DNA
E												
F	N/A		8334 CCR5 DNA		4167 CCR5 DNA		2083 CCR5 DNA		1042 CCR5 DNA		CCR5 DNA NTC	
G	3 HIV	3 HIV	3 HIV	10 HIV	10 HIV	10 HIV	30 HIV	30 HIV	30 HIV	100 HIV	100 HIV	100 HIV
H	300 HIV	300 HIV	300 HIV	1000 HIV	1000 HIV	1000 HIV	3000 HIV	3000 HIV	3000 HIV	HIV NTC	HIV NTC	HIV NTC

Figure 2.6 Plate layout for iCAD assay.

2.8 Statistical analyses

All statistical analyses were performed using R software version 3.3.1 (with earlier datasets) or 3.4.3 (with later datasets). Figure 2.7 provides a summary of the statistical workflow used. Spearman rank order correlations were used to assess what clinical variable could predict study end point HIV-1 DNA. Mixed effect regression models were used to determine what variables would be independent predictors of total HIV-1 DNA decay: including baseline HIV-1 DNA, pre-treatment HIV-1 viral load, absolute CD4 count, age-initiated, time interrupted and study arm as

fixed effects and the participant as the random effect. Decay slopes were described using mixed effect models (participant as random effect and time-treated as fixed effect), separately for very early treated children and interrupted or non-interrupted CHER patients. These mixed effect models enabled us to combine data from all individuals, with an overall good fit, as the random-effects adjust for individual differences in intercepts and slopes, while enabling one to assess the association of fixed effects with the outcome (HIV-1 DNA concentration) across all individuals. While time on treatment is the strongest predictor of HIV-1 DNA concentration in a decay model, other fixed effects were assessed in terms of their influence on the rate of decay. Total HIV-1 DNA decay rates were presented as half-life (t ½) for intuitive comparison between groups and with other published studies.

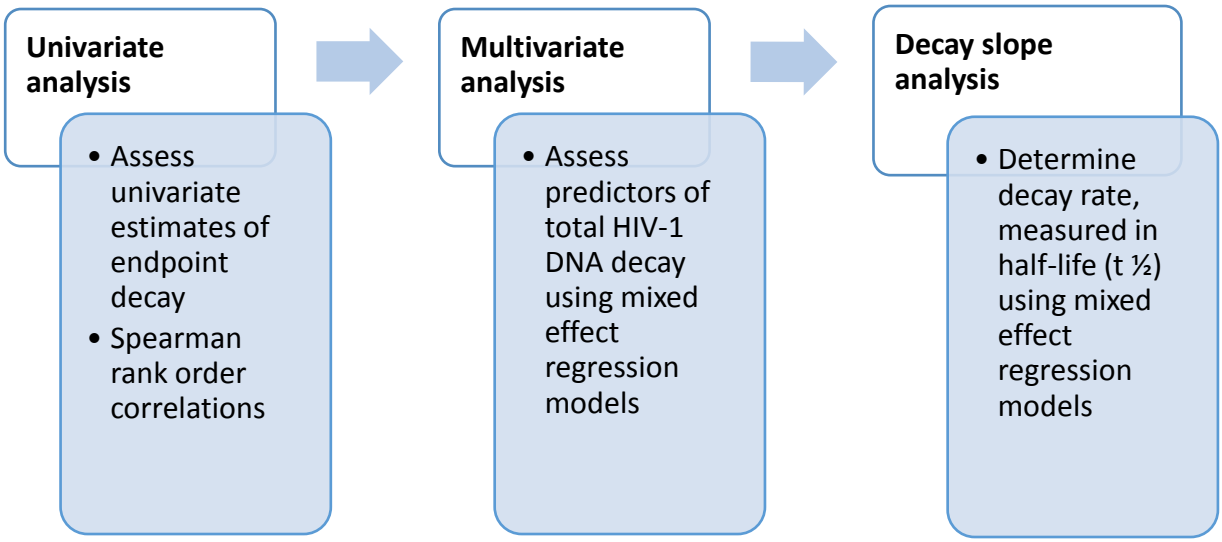


Figure 2.7 Statistical Analysis Workflow

Chapter 3: Results

The results are presented in five main sections. The first section highlights the results from the experiment designed to determine the amount of lymphocytes present in a dried blood spot and the second section focuses on the results from the HIV-1 integrase standard limiting dilution assay. The third section highlights the results from the very early infant diagnosis (VEID) cohort; which included infants who initiated treatment within eight days following birth which corresponds to aim one, objective one of this research project. The fourth section contains the results from the CHER and Post-CHER cohort; which included children who initiated treatment at varying time points according to study specific criteria. The results from this cohort correspond to aim one, objective two; and aim 2 objective 3 of the study. Lastly, in the fifth section the VEID and CHER/Post-CHER HIV-1 DNA decay kinetics results are compared for further discussion in chapter four.

3.1 Total amount of peripheral blood mononuclear cells (PBMCs) in a DBS sample

To determine the amount of peripheral blood mononuclear cells there are in a dried blood spot sample two negative samples were recruited, labelled Neg 1 and Neg 2 with a blood volume of 250 µL and 300 µL, respectively. For each sample a 50µL dried blood spot was made and PBMCs were isolated from the remaining blood volume. DNA was extracted from both DBS and EDTA whole blood PBMC samples and a real-time PCR assay using primers to amplify CCR5 were used to quantify the amount of cells assayed in each sample. The total amount of cells assayed was normalized by sample volume to determine total cells assayed per microliter in DBS and PBMC. These values were used to determine the relative yield of cells assayed in a DBS (PBMC and polymorphs) compared to the number of PBMC recovered from the same volume of whole blood. The relative yields are summarized in Table 3.1. In Neg 2, relatively fewer cells were recovered from DBS when normalized to the same volume of whole blood PBMC. A higher relative cell recovery was expected because a DBS sample also contains polymorphic cells and the observed lower than expected yield could be due to PCR inhibition or poor DNA extraction yield from the DBS sample. In contrast in Neg 1, relatively more cells were recovered in the DBS, likely due the fact that polymorphs are also present in the DBS sample. Apart from variable ratios of PBMC to polymorphs, variable extraction efficiency and PCR inhibition of DBS samples; random variation due to small sample volume could have contributed to the lack of reproducible yield. This high variability observed in these two control samples suggested that we would not be able to use DBS for accurate quantification of pre-treatment HIV-1 DNA load, but we could nevertheless use these samples to qualitatively determine if a particular individual had amplifiable HIV-1 DNA with the iCAD assay primers and probe. This was important especially in cases where HIV-1 DNA loads may decrease rapidly on combination antiretroviral therapy and become undetectable at the first follow-up visit due to low concentration of nucleic acids.

Table 3.1 Percentage PBMCs in a dried blood spot.

Sample name	Sample Type		
Neg1	**DBS**	**PBMC**	**% PBMC assayed in DBS**
Total cells assayed	28153	87733	
Sample volume (µL)	50	200	
T*otal cells per* µL	563,06	438,665	77,91%
Neg2			
Total cells assayed	27551	274712	
Sample volume (µL)	50	250	
T*otal cells per* µL	551,02	1098,848	199,42%

3.2 Limiting dilution results of the HIV-1 DNA integrase standard for the iCAD assay

A limiting dilution experiment was conducted to verify optimal assay performance and to verify that the HIV-1 DNA integrase standard for the iCAD real time PCR assay was prepared correctly and stored at the correct concentration of 1 x10^5 copies per microliter. In this experiment the standard was serially diluted down from the stock concentration above down to one copy per well and loaded onto a real-time PCR (qPCR) run in replicates of 10. As expected all the replicates of the standards at higher concentrations (Std-3 to Std-8) were detected and at the lower concentrations (Std-1 and Std-2) only 5 replicates were detected at the concentration of one copy per well; and nine replicates were detected at the concentration of three copies per well. At the lower concentrations we expect to detect less replicates across the wells due to distribution statistics. The mean cycle threshold decreases at the higher standard concentrations because samples are detected earlier during the real-time PCR run because there is a greater abundance of the target and it is more easily detected as opposed to the lower concentrations which require more amplification cycles to enable detection. Figure 3.1 shows the early detection of higher concentrations (indicated in black and navy blue) versus detection at later cycles at the lower concentrations (indicated in light blue and pink). From the standard curve (Figure 3.2) an efficiency (E-value) of 97.0% was calculated and this correlates to a slope value of -3.396 and the correlation of coefficient value (R^2) was calculated as 0.98.

Table 3.2 Summary of the qPCR limiting dilution assay

Limiting Dilution Assay qPCR results			
Sample	**Dilution (HIV-1 integrase copies per 10µl)**	**Number of replicates detected (out of 10)**	**Mean Cycle threshold (Ct)**
Std-1	1	5	41,97
Std-2	3	9	40,01
Std-3	10	10	38,08
Std-4	30	10	36,89
Std-5	100	10	34,81
Std-6	300	10	33,23
Std-7	1000	10	31,37
Std-8	3000	10	30,05

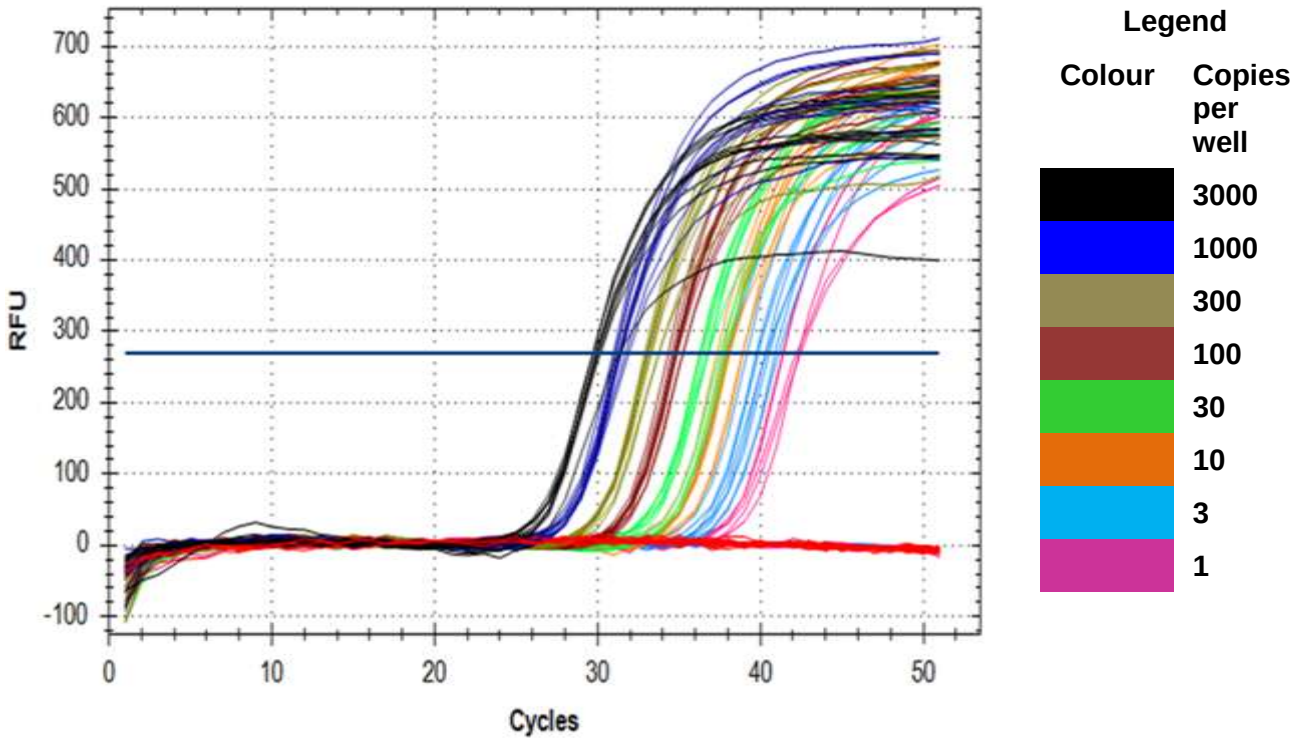


Figure 3.1 Limiting dilution assay amplification plot results

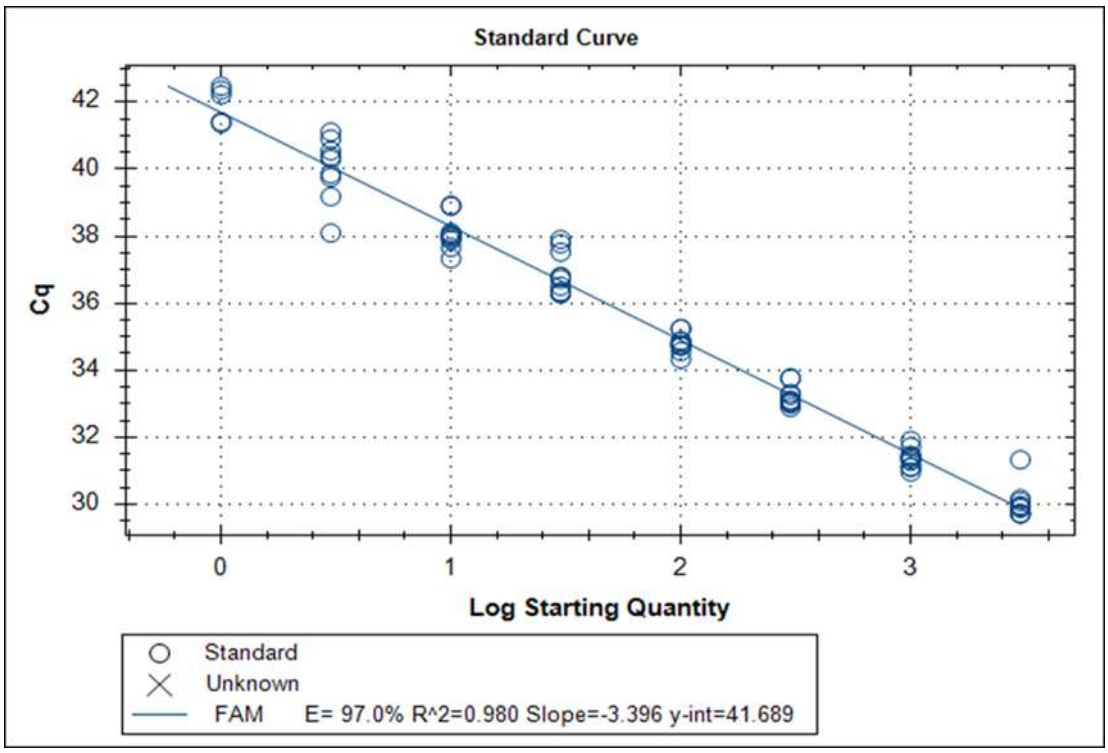


Figure 3.2 Limiting dilution assay standard curve. The open circles represent the different standards from 3000 copies per well to 1 copy per well and multiple circles represent the amount of replicates.

3.3 Very Early Infant Diagnosis (VEID) cohort

3.3.1 iCAD assay characteristics

The iCAD assay was performed on 15 patients, but only 11 were selected for further analysis of which we tested a median of four samples (or time points) per patient for a study period of one year. Table 3.3 provides a summary of the median iCAD value (total HIV-1 DNA copies per million cells) and the mean number of cell equivalents assayed at each time point for all 11 patients. From the summary we observed a decrease in the iCAD value from the baseline time point down to the last visit. The median iCAD value decreased from 714.4 copies per million cells to 48.9 copies per million cells, from baseline to the first study visit, thereafter the median iCAD value decreased by smaller increments until the last study visit. We also observed a large difference between the mean number of cells equivalents assayed at baseline versus the other time points (visit 1 – visit 4) and this is most likely due to the difference in the sample type because all baseline samples were DBS samples and the study visits were PBMC samples.

Table 3.3 Summary of iCAD assay characteristics for the VEID cohort

iCAD assay participants		
Number of patients tested	15	
Number of samples tested per patient* (Median ([QR])	4 [4-5]	
iCAD assay summary		
Visit number	**Median iCAD value (total HIV-1 DNA copies per million cells) [IQR]**	**Mean number of cells equivalents assayed**
Baseline	717.4 [516.5-1722.5]	131619
Visit 1	48.9 [0.0-509.3]	579557
Visit 2	11.4 [0.0-107.0]	556598
Visit 3	18.0 [0.0-35.05]	703159
Visit 4	0.3 [0.0-6.6]	627513

* Includes only the 11 participants selected for further HIV-1 DNA and HIV-1 RNA kinetics analysis. Sample also refers to the number of time points tested.

3.3.2 Participant characteristics

A total of 15 participants were selected for the study because they had a baseline sample available and two on-treatment samples. However, four participants were excluded from HIV-1 DNA and HIV-1 RNA decay analysis. Two infants had no detectable plasma HIV-1 RNA and total HIV-1 DNA, and another two only had detectable HIV-1 RNA. Table 3.4 is a summary of the remaining 11 infants who initiated ART between 0-8 days (median 4 days) and with an average baseline HIV-1 RNA of 3.9 log copies/mL. Seven infants were classified as suppressed (plasma HIV-1 RNA load <100 copies/mL after 6 months on ART) and four were classified viremic. All participants were part of a child PMTCT program consisting mainly of a dual regimen (NVP. AZT), but two were initiated on immediate combination antiretroviral therapy.

Table 3.4 Participant characteristics for the VEID cohort

Participant characteristic	Total number of participants (n=11)
Child PMTCT drugs	NVP,AZT (n=7)
	NVP (n=1)
	Immediate cART (n=2)
	AZT,3TC (n=1)
Initial ART regimen*	AZT,3TC, NVP (n=11)
Age at ART initiation (days); median(IQR)	4.0 (3.0– 7.0)
Average Baseline HIV-1 RNA (log copies/mL)	3.9 (2.6-4.7)
Virologic status (6 months after ART)	Suppressed (n=7); Viremic (n=4)
Study period (months); median(IQR)	6.9 (3.5-11.6)

*NVP replaced by LPV/r at corrected age of 42 weeks; AZT replaced by ABC at approximately 3 months of age

3.3.3 HIV-1 DNA and HIV-1 RNA kinetics

Of the seven suppressed infants, HIV-1 RNA was undetectable (<100 copies/mL for 200 microliter input) in three infants within 3.4 months and in one participant HIV-1 RNA reached a 120 copies/mL at 3.5 months. In the remaining three the first undetectable plasma HIV-1 RNA loads were recorded between six and seven months. Total HIV-1 DNA decay kinetics were characterised in seven suppressed participants. Pre-treatment DBS samples were used to determine if there was a detectable baseline HIV-1 DNA load, but we could not characterize the decay from baseline to first on-ART time point. Total HIV-1 DNA decay was measured from the first time point to last time point for the study period (Figure 3.3).HIV-1 DNA decay slopes showed a good log-linear correlation with a mean half-life of 64.8 days and a conditional R^2 (95% CI) of 0.77 (0.53-0.91). At the end of the study period, six out of seven participants' total HIV-1 DNA loads decreased to less than ten copies per million PBMCs. One participant of the six had an undetectable HIV-1 DNA load at the second study visit, 3.5 months on ART (participant S9) and another participant (participant S3) had a detectable HIV-1 DNA load at 11.6 months, but it was unquantifiable by our assay. Furthermore, one of the seven participants (participant S6) who had delayed viraemia suppression had a total HIV-1 DNA load remaining above 10 copies per million PBMCs, only dropping down to 47.8 copies per million PBMCs at the end of the observation period for this participant. Of the participants classified as viraemic, two participants (V3 and V5) had early undetectable HIV-1 DNA at their first study visits with subsequent viraemia. Participant V5 had continued viraemia along with an increase in HIV-1 DNA whereas participant V3 had a single episode viraemia with HIV-1 DNA remaining undetectable. Participant V1 had an undetectable plasma HIV-1 RNA load at a second study visit, but HIV-1 DNA remained detectable at that time point and continued to increase thereafter. Participant V4 had a decrease in HIV-1 DNA load despite on-going detectable viraemia.

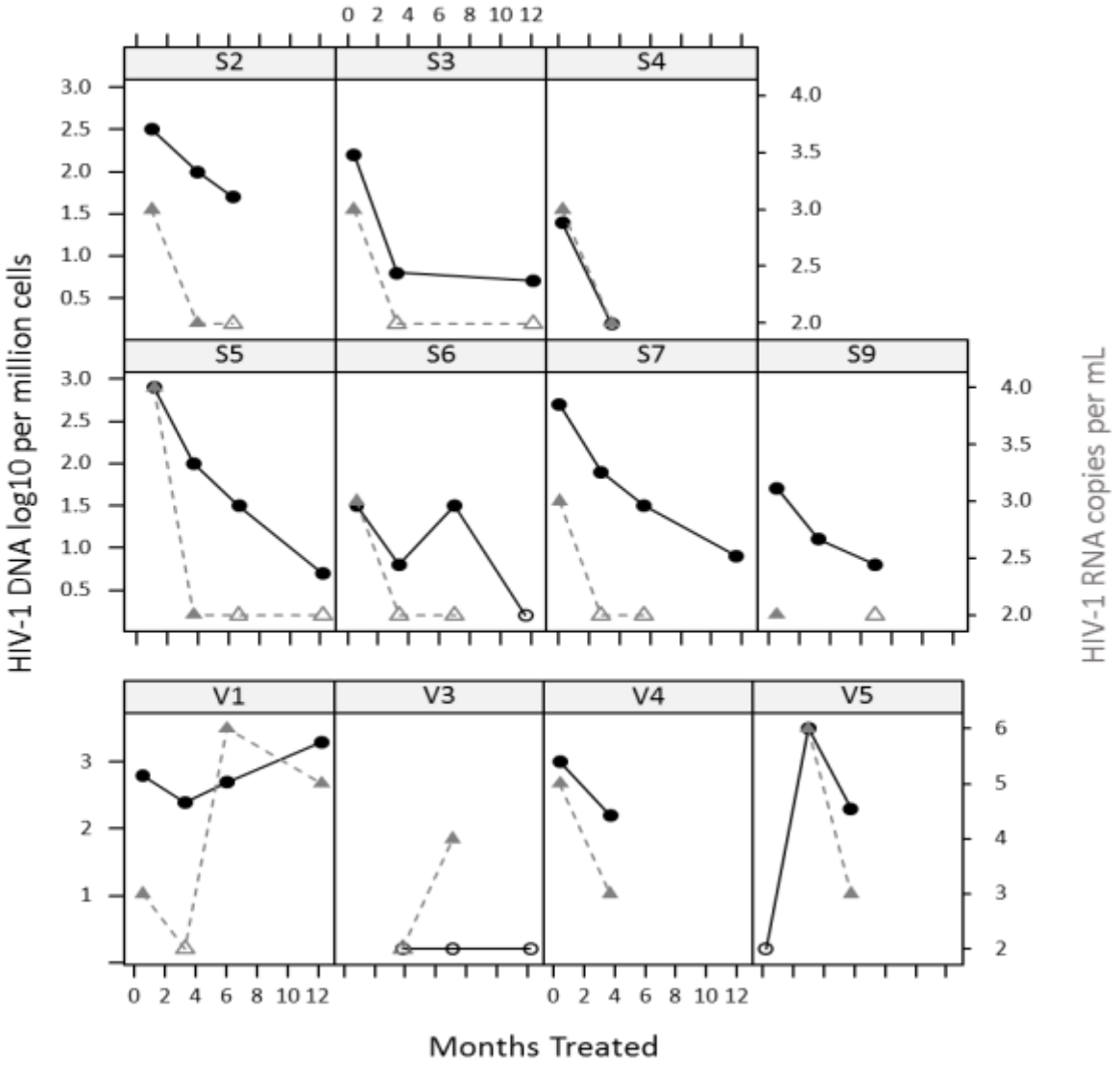


Figure 3.3 HIV-1 DNA and RNA kinetics in the VEID cohort. "S" indicates suppressed infants and "V", viremic infants. HIV-1 DNA loads are denoted by black circles and plasma HIV-1 RNA loads are denoted by grey triangles. Open icons indicate HIV-1 DNA or HIV-1 RNA below the limit of detection: plasma HIV-1 RNA load was censored at <100 copies per mL and HIV-1 DNA loads at <3 copies per million PBMCs. HIV-1 DNA and RNA decay slopes were determined in the seven suppressed infant s.

In summary, we observed a rapid viral suppression after treatment started very early after infection. Six out of seven participants' total HIV-1 DNA load decreased to less than 10 copies per million PBMCs, with one participant having delayed viraemia suppression. Overall there was a fast decay rate (mean t ½ of 64.8 days).

3.4 Children with HIV early antiretroviral therapy (CHER) cohort and Post-CHER cohort

3.4.1 iCAD assay characteristics

The iCAD assay was performed on 40 patients from the CHER and Post-CHER cohort. We tested a median of five samples (or time points) per patient for a study period of up to 10 years. Table 3.5 provides a summary of the median iCAD value (total HIV-1 DNA copies per million cells) and the mean number of cell equivalents assayed at each time point for all 40 participants. The mean number of cell equivalents assayed across all time points was relatively the same. The median iCAD value decreased over time from baseline to the last study visit. We observed a similar trend as in the VEID cohort, where there was a large decrease in the median iCAD value from the baseline time point (1601.5) to the first sample six months (90.4) after baseline. The baseline sample taken prior to re-initiation of therapy only applies to participants who were therapy interrupted. After six months the median iCAD value decreased more gradually until the last study visit time point.

Table 3.5 Summary of iCAD assay characteristics for the CHER/Post-CHER cohort

iCAD assay participants		
Number of patients tested	40	
Number of samples tested per patient (Median [IQR])	5 [5-7]	
iCAD assay characteristics per visit		
Visit ID	**Median iCAD value (total HIV-1 DNA copies per million cells) [IQR]**	**Mean number of cells equivalents assayed**
Baseline	1601.5 [739.75-3271.20]	527462
Baseline prior to re-initiation of therapy*	831.9 [313.45-1372.90]	591327
6 months	90.4 [43.20-424.80]	522851
12 months	93.0 [61.125-343.60]	503074
18 months	91.0 [18.0-232.20]	484458
7/8 years (Post-CHER Visit 1)	21.2 [6.75-39.95]	461445
9/10 years (Post-CHER Visit 4)	22.5 [0.75-46.0]	505591

*Only applies to patients who were therapy interrupted

3.4.2 Participant characteristics

A total of 40 participants were tested, but only 31 were selected for further analyses. These individuals were viraemia-suppressed (viral load less than or equal to 200 copies per millilitre across two or more time points) and met the criteria (stated in section 2.3.3) which made them suitable candidates to study HIV-1 DNA decay and potential predictors associated with decay. Table 3.6 provides an overview of the participant characteristics for this cohort. Fifteen of the participants came from Part A, Arm 2 of the CHER study; eight came from Part A, Arm 3; six came from Part A, Arm 1 and two from Part B, Arm 2 of the study. Furthermore, eight of the 31 were never interrupted and had continuous antiretroviral therapy. Two participants who were never interrupted were initially recruited into a study arm with a scheduled interruption period, but due to clinical reasons they were continued on therapy. All participants were initiated on a triple regimen of zidovudine (AZT), lamivudine (3TC) and lopinavir-ritonavir (LPV/r), except one who started with stavudine (D4T) instead of zidovudine as part of their treatment regimen. The median age of ART initiation was 58 days and for the subset that was never interrupted the median ART initiation age was 156.5 days. The median CD4 percentage was 17.9% and the absolute CD4 count 691 cells per microliter at baseline or prior to continuous therapy. At the end of this study period the median age of the participants at the last sample time point was 9.8 years, the median CD4 percentage was 37% and the absolute CD4 count 948 cells per microliter.

Table 3.6 Participant characteristics for the CHER and Post-CHER cohort. CHER study arms. A1: Part A, Arm 1; A2: Part A, Arm 2; A3: Part A, Arm 3; B1: Part B, Arm 2.

Participant characteristic	All(n=40)	Subset: Viraemia-suppressed (n=31)	Subset: Viraemia-suppressed and never interrupted(n=8)
Treatment regimen	AZT, 3TC, LPV/r (n=36); AZT, 3TC, LPV/r then ABC, 3TC, LPV/r (n=1); D4T, 3TC, LPV/r (n=1); ABC, AZT, 3TC then ABC, 3TC, LPV/r(n=1); ABC, DDI, NVP then AZT,3TC,EFV then ABC, 3TC, LPV/r (n=1)	D4T, 3TC, LPV/r (n=1); AZT, 3TC, LPV/r (n=30)	AZT, 3TC, LPV/r
Study arms	A1(n=8) A2(n=18) A3(n=10) B2(n=4)	A1(n=6) A2(n=15) A3(n=8) B2(n=2)	A1(n=6) A3(n=2)
Interruption status	Interrupted (n=27) Never interrupted (n=13)	Interrupted (n=23) Never interrupted (n=8)	- Never interrupted(n=8)
Age ART initiated (days); median(IQR)	64(52-82.5)	58 (51.5-77)	156.5 (110.3-256.8)
CD4% nadir *; median (IQR)	17.6% (14.5-23.6%)	17.9 (15.15-24.15)	16.9 (14.0-19.1)
Absolute CD4 count nadir cells/microliter *; median (IQR)	676.5(478.5- 996.5)	691 (505-1038.5)	505(440.5-759.5)
Time interrupted (days); median (IQR)	98(0-254)	133 (0-247)	_
Age at last sample (years); median (IQR)	10(8.7-10.6)	9.8(8.8-10.6)	9.5 (9.3-10.2)
CD4% at last sample	37% (34-42%)	37 (34-43)	40 (32.75-43.5)
Absolute CD4 (cells/ microliter) at last sample	943(801-1089)	948 (841-1134.5)	981 (955.25-1133.75)

ART: antiretroviral therapy; IQR: interquartile range; CHER:
*(before continuous treatment); Duration of interruption was

3.4.3 Fitting decay HIV-1 DNA decay curves

Most individuals did not have a fixed log linear decay across time and HIV-1 DNA decay rate seemed to decrease over time. In Figure 3.4 the time treated (x-axis) was transformed to square root of days treated and provided the best fit for the data with a conditional R^2 of 82% (compared to a conditional R^2 of 73% without square root transformation of time treated).

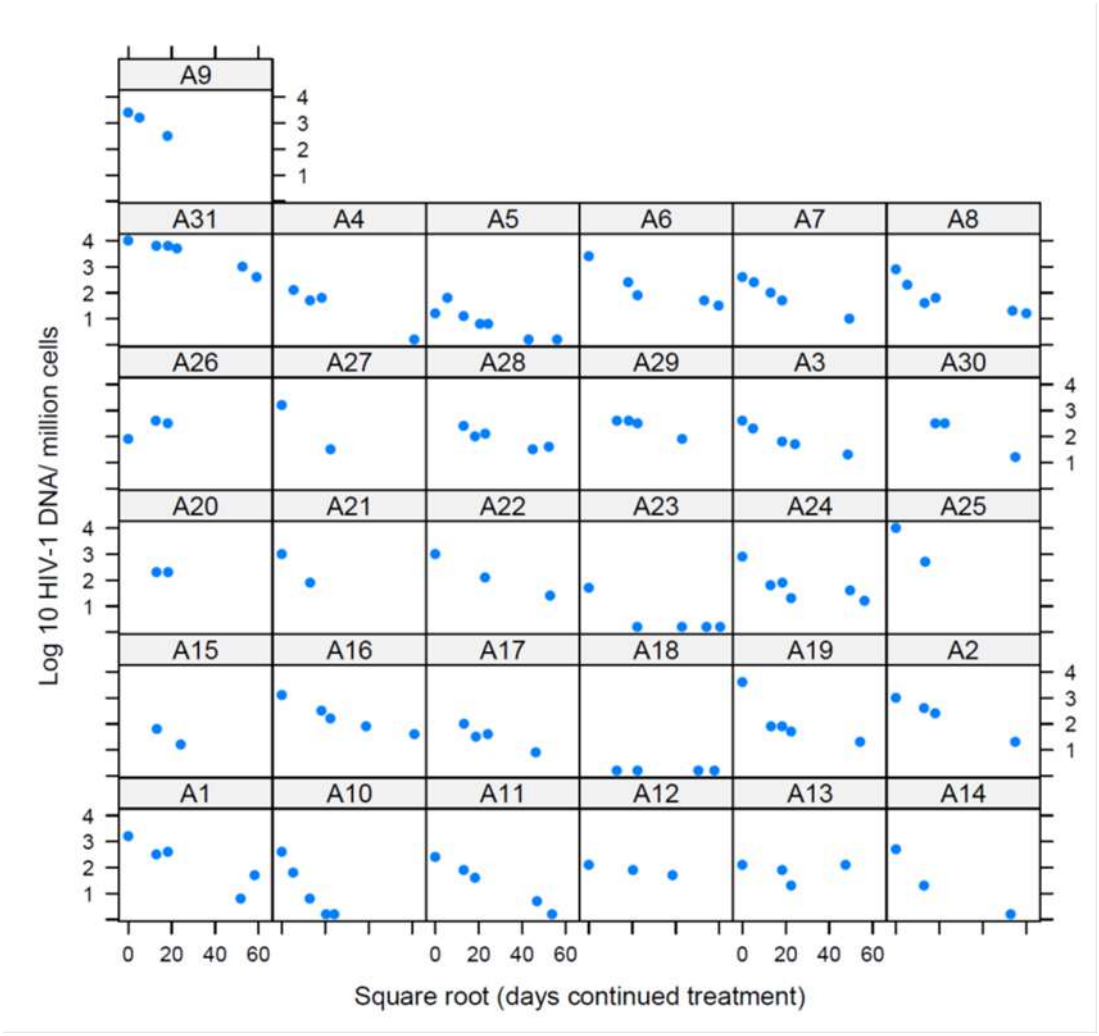


Figure 3.4 HIV-1 DNA decay: cubic model of 31 viraemia-suppressed participants.

3.4.4 Impact of therapy interruption

Wilcoxon rank sum tests were used to assess pre-interruption, post-interruption and endpoint HIV-1 DNA levels. This is a non-parametric statistical measure was used as the data is not normally distributed and accounts for the variability seen among participants. Figure 3.5 shows that there was no significant difference between pre-interruption log HIV-1 DNA versus post-interruption log HIV-1 DNA (W = 353, P= 0.18). In contrast, there was a significant difference in post-interruption HIV-1 DNA levels versus endpoint log HIV-1 DNA (W= 558, p<0.001). The impact of therapy interruption on the CD4 percentage and absolute CD4 count at the end of the study was

determined using a Spearman rank correlation test by plotting days interrupted against CD4 percentage and absolute CD4 count at the end of the study. This test is used to assess the relationship between two variables and can indicate the strength of the relationship and whether it is a negative or positive association. In figure 3.6 we see no significant correlation between both parameters and period of therapy interruption. Furthermore, there was no significant correlation between the period of interruption and study endpoint HIV-1 DNA (Spearman rho = 0.35; p =0.055). However, there is a trend toward higher HIV-1 DNA levels and longer periods of interruption (Figure 3.7a). Additionally, two arbitrary categories of interruption were compared, interruption for less than 58 days (lower tertile) versus interruption for at least 58 days. The lower tertile (<58 days) had a significant Wilcoxon Rank Sum test p-value of 0.18 and may suggest that shorter periods of interruption may not have an effect on total HIV-1 DNA level at end of the study (Figure 3.7 b).

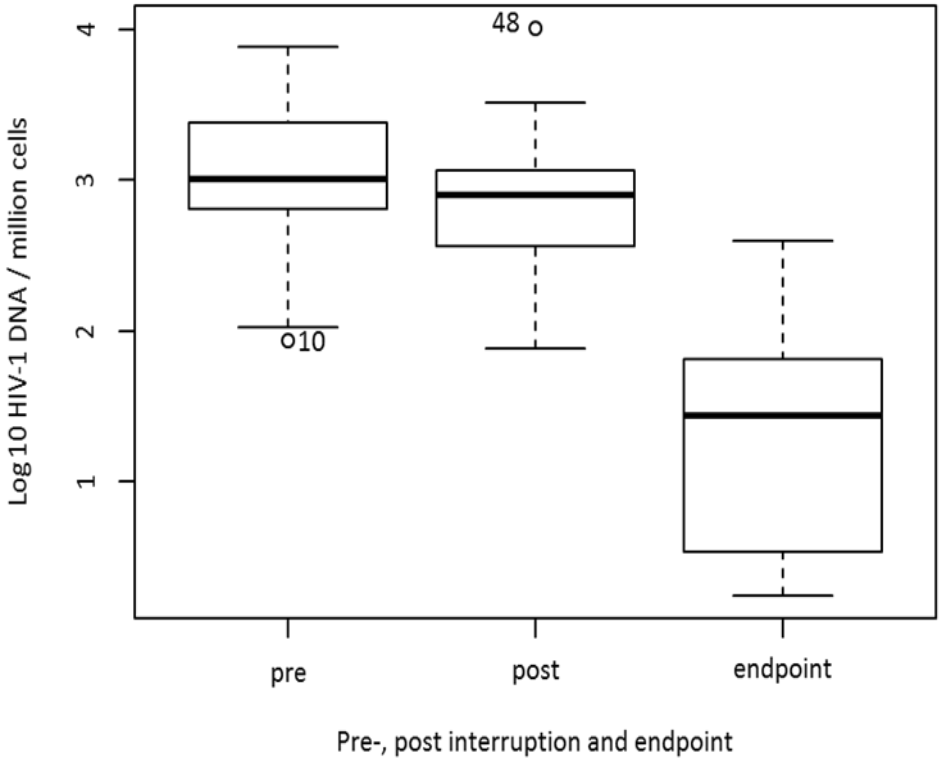


Figure 3.5 Pre- and post-interruption HIV-1 DNA and endpoint HIV-1 DNA (n=27).

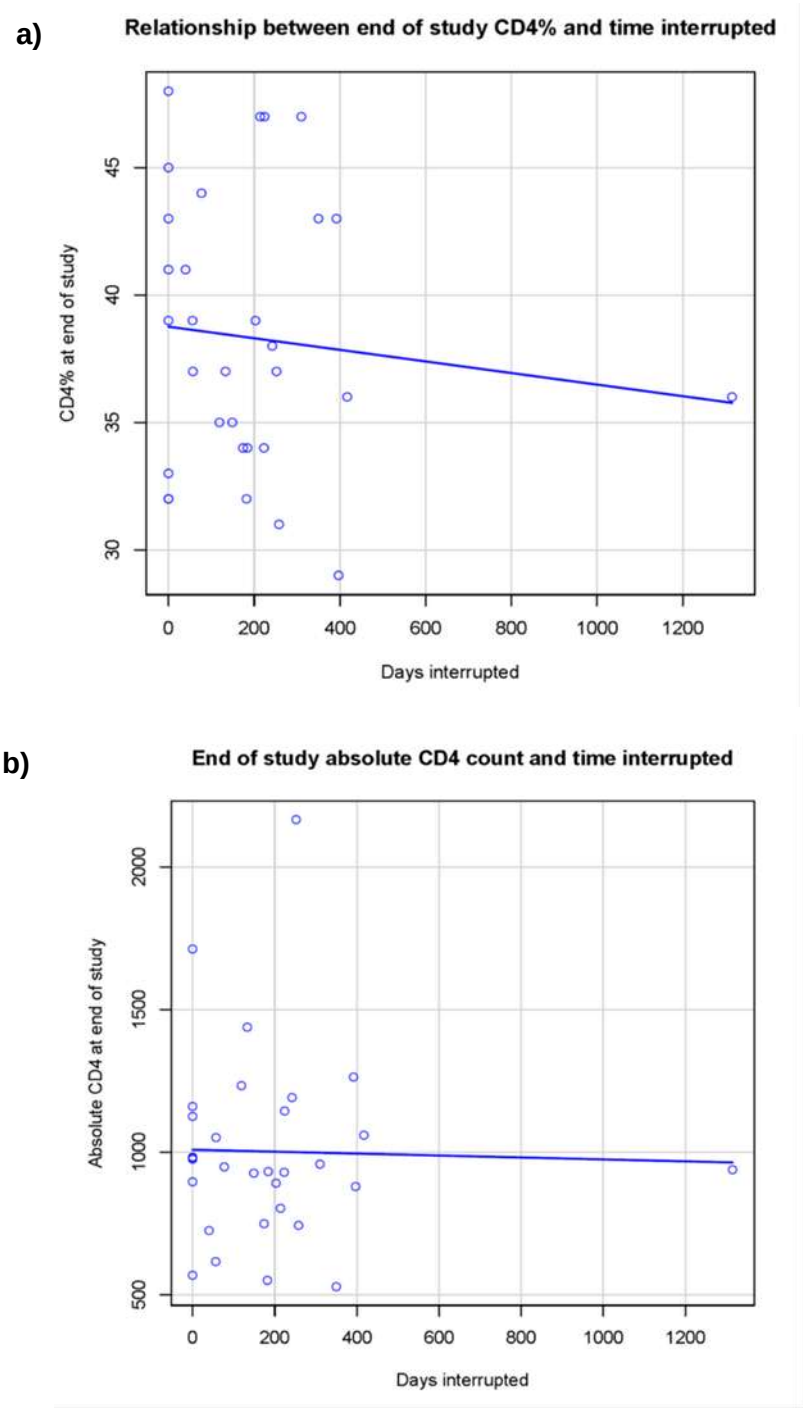


Figure 3.6 Impact of therapy interruption on CD4% and absolute CD4 count at the end of the study. a) The Spearman rank correlation (rho) shows no significant correlation between period of interruption in days versus the CD4% at the end of the study (rho = 083; p = 0.7). b) Spearman rho = -0.057; p = 0.76, therefore there is no significant correlation between period of interruption and absolute CD4 count.

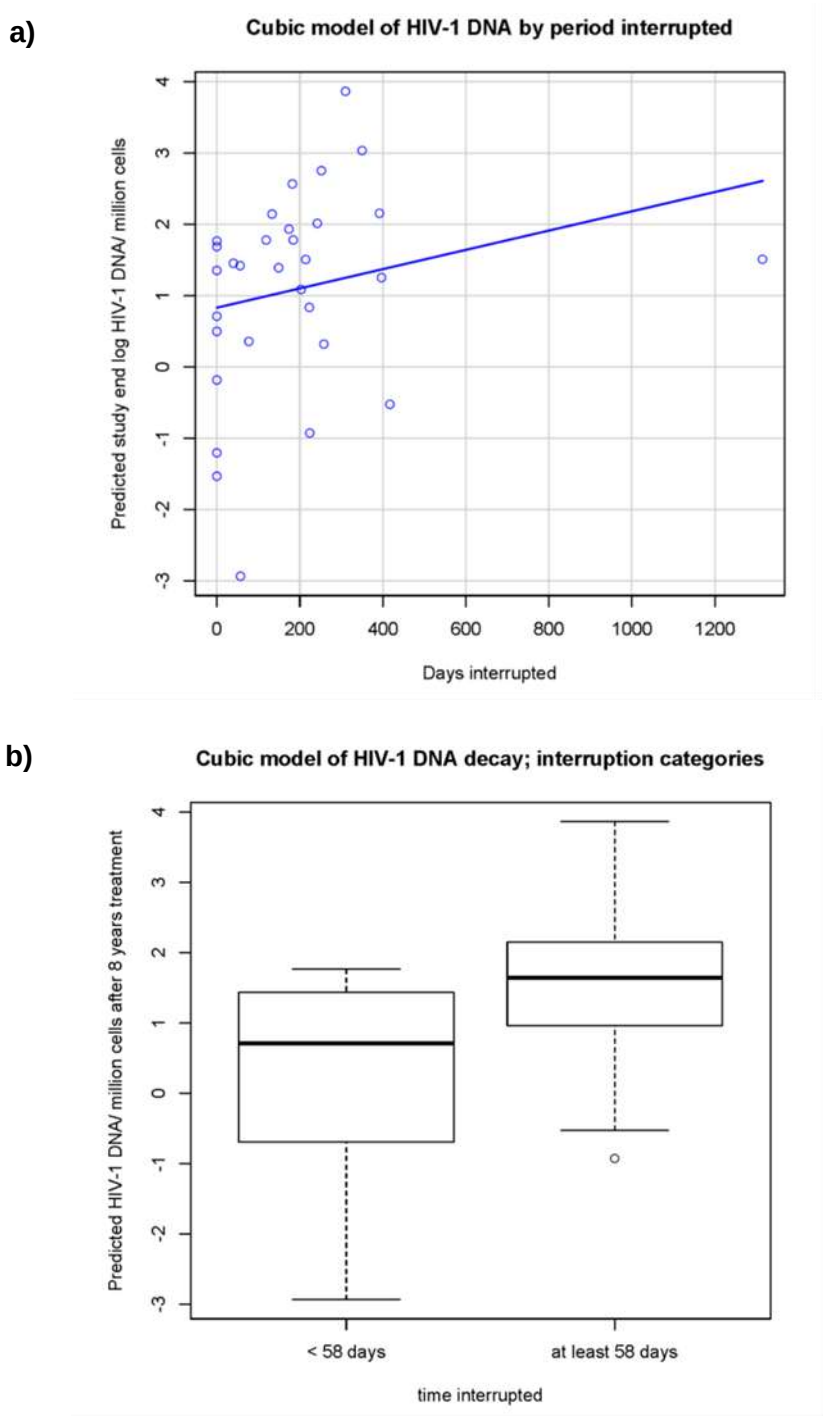


Figure 3.7 Impact of therapy interruption on study endpoint HIV-1 DNA. a) There is no significant correlation between the period of interruption and study endpoint HIV-1 DNA (Spearman rho = 0.35; p =0.055). b) The lower tertile (<58 days) had a significant Wilcoxon Rank sum test p-value of 0.18.

3.4.5 Predictors of total HIV-1 DNA decay

HIV-1 DNA level before treatment (p<0.0001), increased HIV-1 DNA concentration over a period of interruption (p<0.01) and a higher absolute CD4 count pre-therapy (p = 0.01) predicted a slower decay (Table 3.7). A faster decay rate was observed in participants from the Arm 3 group of the

study who had a longer initial period of therapy (96 weeks) before the start of an interruption period, but two of these children were not interrupted. However, the association remained significant after controlling for interruption (p = 0.02). Other variables such as CD4% nadir before treatment (p=0.38), pre-treatment HIV-1 RNA (p=0.051), age-starting ART (p=0.73), time-interrupted (p=0.76) and viraemia copy years during interruption (p=0.98) were not found to be independent significant predictors of HIV-1 DNA decay.

Table 3.7 Mixed effect model of log HIV-1 DNA decay against the square root of time on continued treatment

Fixed effects	Estimate	Std.Error	degrees of freedom	t-value	p-value
(Intercept)	1.2487	0.5750	18.4046	2.1710	0.043
Square root of days treated	-0.0274	0.0019	102.5555	-14.5950	**<0.0001**
CD4% nadir before treatment	-0.0131	0.0146	17.2068	-0.8950	0.38
Absolute CD4 nadir before treatment	0.0006	0.0002	17.4915	2.7870	**0.01**
Pre-treatment HIV-1 DNA level	0.8016	0.1109	16.1337	7.2260	**<0.0001**
Change in HIV-1 DNA levels over interruption	0.8508	0.2521	21.6229	3.3750	**<0.01**
pre-treatment HIV-1 RNA level	-0.1719	0.0817	16.4751	-2.1030	0.051
Viraemia copy years during interruption	-0.0021	0.0760	18.9584	-0.0280	0.98
Age ART started	0.0003	0.0009	50.4246	0.3500	0.73
Time interrupted	0.0003	0.0009	19.3851	0.3050	0.76
Continuous vs. interrupted therapy	0.0525	0.2935	15.8483	0.1790	0.86
Study arm A2 (vs. A1)	-0.4774	0.3919	19.1950	-1.2180	0.24
Study arm A3 (vs. A1)	-0.8794	0.3554	20.3218	-2.4740	**0.022**
Study arm B2 (vs. A1)	-0.0192	0.4420	20.5826	-0.0440	0.97

3.5 Comparison of total HIV-1 DNA decay in the VEID and CHER cohorts

The first derivative (slope) of $\log_{10}$ HIV-1 DNA against the square root of time was used to calculate point estimates of total HIV-1 DNA half-life (t ½) in eight viraemia-suppressed participants who were never therapy interrupted and 23 viraemia-suppressed participants who were previously interrupted before starting continuous therapy at a median of 20 months post-interruption. The

square root of time was treated as a fixed effect and participant ID was treated as a random effect in a cubic mixed effect model. The conditional R^2 (95% CI) values for the HIV DNA decay curve was 0.82 (0.65-0.93) for the VEID infants (early ART start, n=7), 0.85 (0.67-0.94) for the CHER children (late ART start, n=8) and 0.79 (0.68-0.86) for the CHER children with previous interrupted ART (n=23). At 6 months of treatment, in the eight never-interrupted viraemia-suppressed individuals, who started treatment around five months of life the t ½ of HIV-1 DNA was 9.2 (95% CI: 7.4-12.1) months, significantly slower than the 2.7 months (95%CI: 2.1-3.8) t ½ seen in seven viraemia-suppressed infants who started ART around four days after birth (p<0.01) (Figure 3.8 a-b). The observed decay rate in previously interrupted CHER children (n=23) was 9.6 months (95%CI: 7.6-12.6) t ½ (Figure 3.8 c); which was similar to the half-life observed in 8 CHER children who were never interrupted, but started later on ART (p=0.81; Wilcoxon Rank Sum Test)

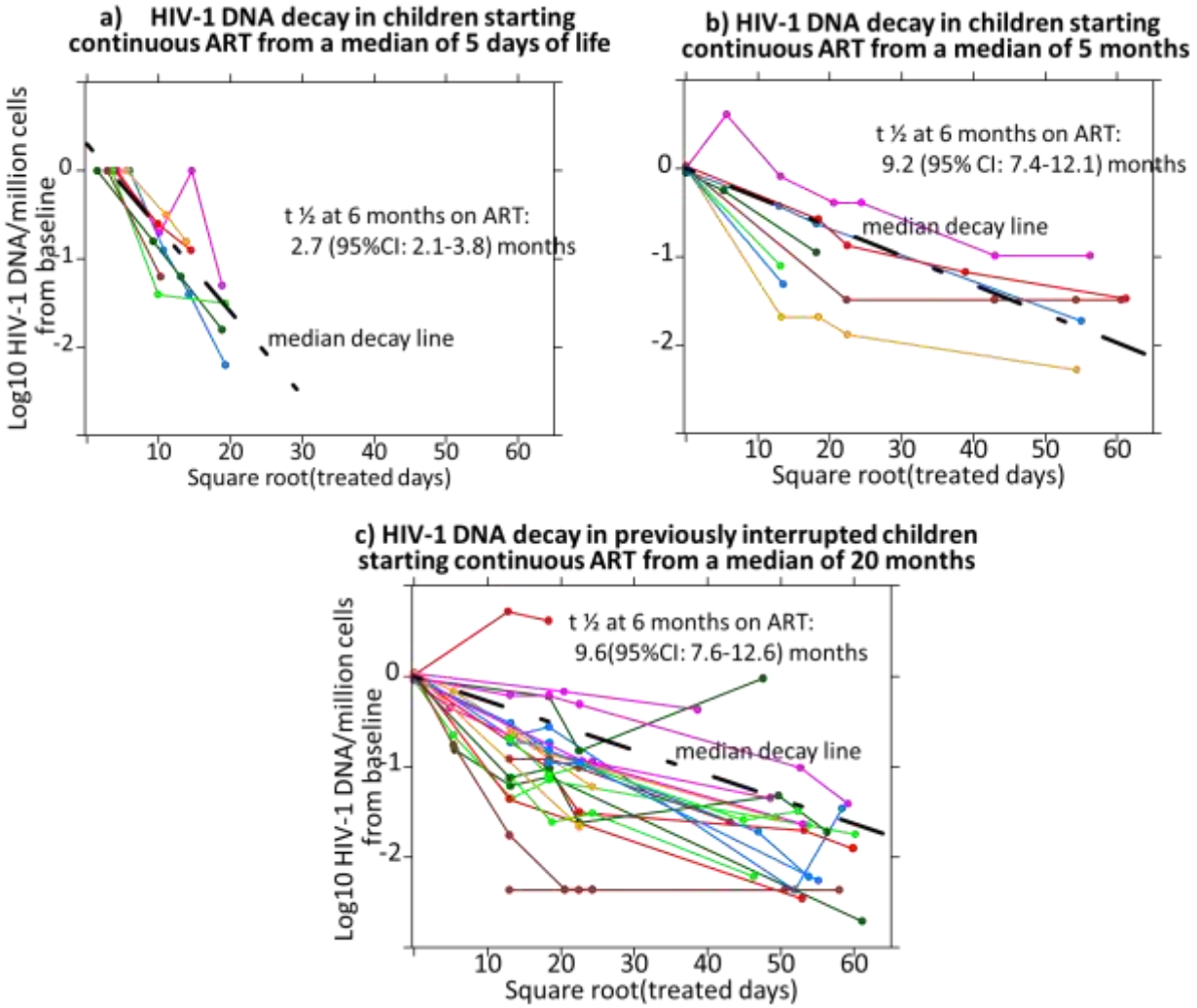


Figure 3.8 Comparison of total HIV-1 DNA decay rates in participants who were initiated early on ART versus later ART initiation and previously interrupted participants.

Chapter 4: Discussion

4.1 Assay validation

4.1.1 Variable cell yield from dried blood spots

Cellular DNA recovery from DBS samples were variable, when quantified using a real-time PCR assay targeting the human CCR5 gene. From the two samples we could retrieve that had sufficient volumes, one sample (Neg 2) had relatively fewer cells recovered from DBS when normalized to the same volume of whole blood PBMC. A relative higher cell recovery was expected because a DBS sample also contains polymorphonuclear leucocytes (predominantly neutrophils), and this lower than expected yield could be due to PCR inhibition or poor DNA extraction yield from the DBS sample. In contrast in the Neg 1 sample, relatively more cells were recovered from the DBS sample and this is likely due the fact that polymorphs are also present in the DBS sample. Apart from the variable ratios of PBMC to polymorphs, variable extraction efficiency and PCR inhibition of DBS samples; random variation which is expected to be higher when extracting few cells (due to small sample volume) could have contributed to the lack of reproducible yield. This high variability observed in these two control samples suggested that we would not be able to use DBS samples to accurately quantify baseline HIV-1 DNA load, but could nevertheless use DBS samples to qualitatively determine if HIV-1 DNA from a particular individual was detectable with the iCAD assay primers and probe. This was important to show as HIV-1 DNA loads may decrease rapidly in infants receiving combination antiretroviral therapy and could have become undetectable at their first study visit when EDTA blood was collected.

4.1.2 Accurate quantification of integrase HIV-1 DNA standard for iCAD assay

At HIV-1 standard dilutions of three copies per well and one copy per well, respectively 9 out of 10 and 5 out of 10 replicates were detectable. This agreed with the expected proportion of positives (or expected hit rate) according to the Poisson distribution, which provides support that the HIV-1 integrase standard used for the iCAD assay was prepared and stored at the correct concentration of 1 $x10^5$ copies per microliter. A valid real-time PCR run should have an efficiency value (E-value) between 90%-110% which corresponds to a slope of between -3.58 and -3.10. The correlation of coefficient (R^2) should be as close to 1 as possible, for this study a R^2 above 0.95 was considered acceptable. These criteria were used for all real-time PCR assays in this thesis. The limiting dilution experiment standard curve (Figure 3.2) shows a slope value of -3.396 which corresponds to an efficiency (E-value) of 97.0% and the correlation was (R^2) was 0.98. This verifies that the qPCR run was acceptable according to the above criteria and results from the limiting dilution assay were valid.

4.2 Infants diagnosed and treated very early after birth (VEID cohort).

4.2.1 Rapid HIV-1 DNA and RNA decay after very early ART initiation

In infants with rapid plasma HIV-1 RNA suppression, who reached less than 100 copies/mL within 3.4 months on ART, total HIV-1 DNA became undetectable in three out of eleven infants at 6 days, 3.5 months and 4 months, respectively. The initial phase of decay between ART initiation and first study visit could not be assessed because HIV-1 DNA could not be accurately quantified on DBS samples from pre-treatment. The decay rate of total HIV-1 DNA in PBMC samples (taken from the first study visit onwards) was 65 days (t ½). In a study where adults were treated in the acute phase of HIV infection (Fiebig stage I and II) a rapid decay was observed with a half-life of 21 days in the first two weeks thereafter a much slower decay was observed at a half-life of 198 days (Ananworanich *et al.*, 2016). In children initiated on therapy within two months of birth total HIV-1 DNA decay at 24 weeks of life was 53 days (t ½) and a decay rate of 124 days (t ½) was seen between 24 and 48 weeks of life (Uprety *et al.*, 2015). Another study in children who initiated therapy within three months after birth reported a half-life of 107 days in the first year of life (McManus *et al.*, 2016). Therefore, HIV-1 DNA decay observed in our study was faster than what was seen in other similar studies.

The possible reasons for rapid HIV-1 DNA decay within the blood of infants treated shortly after birth could be due to a lower starting quantity of infected cells (pre-treatment) as a result of maternal ART and infant prophylaxis. Another reason could be the rapid loss of cells with unintegrated virus (Ananworanich *et al.*, 2016) and a high CD4 turnover with a large proportion of short-living infected CD4 cells that decay rapidly (Schönland *et al.*, 2003). Furthermore, a large proportion of infected cells could contain integrated HIV-1 DNA that may be defective and contain deletions and this non-intact virus could result in a negative (undetectable) result on our iCAD assay (Bruner *et al.*, 2016).

4.2.2 Implications of rapid decay for infant HIV-1 diagnosis

Rapid early phase HIV-1 RNA and DNA decay poses a diagnostic challenge in infants receiving extensive neonatal prophylaxis. Additionally, starting concentrations of HIV-1 DNA or RNA, in treated infants may be very low due to maternal ART (effectively intra-uterine treatment of infected babies), and possibly some ARV absorption through breastfeeding (Waitt *et al.,* 2015) that may together contribute to suppression of viral replication after infection. HIV-1 DNA and RNA could be so low that it could result in false negative diagnostic tests. It is therefore crucial that a definitive diagnosis is established as soon as possible after birth before HIV-1 DNA or plasma HIV-1 RNA become undetectable.

4.3 Children with HIV early antiretroviral therapy (CHER) and Post-CHER cohort

4.3.1 Relatively slower Total HIV-1 DNA decay with later ART initiation

In the eight viraemia-suppressed children who were never therapy interrupted and who started ART around 5 months of life; the decay of HIV-1 DNA was much slower than in children who started around a median of 4 days of life, 9.2 months (t ½) versus 2.7 months (t ½), respectively. Other studies found lower HIV-1 DNA levels in children who started ART before 3 months (Kuhn *et al.*, 2018; Luzuriaga *et al.*, 2014; Martínez-Bonet *et al.,* 2015; McManus *et al.*, 2016; Van Zyl *et al.,* 2015), before 6 months (Ananworanich *et al.*, 2014) and before one year of age (Foster *et al.,* 2017) versus later ART initiation. A recent article from a European cohort also showed that earlier ART start and better virologic suppression in infants was associated with lower total HIV-1 DNA at age 4-11 years (Tagarro *et al.*, 2018). An earlier study also reported a faster decrease in HIV-1 DNA levels within in a group of infants who achieved virologic suppression within first year of life versus infants who achieved virologic control between 1-5 years of age (Uprety *et al.,* 2017). However, when assessing HIV-1 DNA decay a study investigating longitudinal samples found no difference in the rate of decay (McManus *et al.*, 2016) in children starting ART earlier than three months or later. This suggests that there may be a very early window when the pool of HIV-1 infected cells is more labile.

There was no significant difference in HIV-1 DNA decay rates between the group of CHER children who started treatment around 5 months(t ½ of HIV-1 DNA 9.2 (95% CI: 7.4-12.1) months) ($p<0.01$) and in the 23 who started around a median of 1.8 months, who interrupted for a median of 7 months, and reinitiated at a median of 20 months (HIV-DNA t ½ 9.6(95%CI: 7.6-12.6) months post-interruption ($p=0.81$). This is in contrast to another study that investigated the impact of short term interruption on HIV-1 DNA decay in infants who initially started continued ART at a median of 4.8 months with a subsequent short interruption period for a median of 106 days. Following the resumption of ART at around 15 months post-interruption and following plasma HIV-1 RNA re-suppression, they found a slower HIV-1 DNA decay rate after interruption versus the initial decay rate observed after initiating continued ART (Pankau *et al.,* 2018). However, in the CHER trial children who electively started early were randomized to interruption, whereas those who started based on clinical and immunological criteria generally started later and were not interrupted. It was therefore not possible to isolate the effects of interruption and early start on HIV-1 DNA decay amongst the CHER patients.

4.3.2 Predictors associated with decay

In this study the strongest predictors of a slower decay rate were pre-treatment HIV-1 DNA levels and the change in HIV-1 DNA before versus after interruption. Higher pre-treatment HIV-1 DNA levels and an increase in HIV-1 DNA over the period of interruption resulted in a slower decay

following the restart of continuous therapy. Patients were reinitiated on treatment based on CD4 measures and other clinical criteria, patients with more rapid progression during interruption were initiated faster. This could have counteracted any increased replenishment with longer periods of interruption. Participants with an initial treatment period of 96 weeks (prior to interruption) had a faster decay rate and this suggests an early period of prolonged therapy may be valuable in limiting HIV-1 persistence.

4.3.3 No significant changes in total HIV-1 DNA levels pre- and post-interruption

Overall, there was no significant difference in HIV-1 DNA levels pre-interruption versus post-interruption. Only when the lower tertile of period of interruption (<58 days) was compared to the rest of population, was there a significant lower total HIV-1 DNA level at the end of the study period. In a cohort of chronically HIV-1 infected adults undergoing at least one short analytical treatment interruption (ATI), for a period of six weeks or less, there was no difference found in HIV-1 DNA levels pre-ATI and post-ATI following resumption of ART and plasma HIV-1 RNA re-suppression (Papasavvas *et al.,* 2018). Another study conducted in adults with a median ATI phase of 57 days also found no lasting effect of interruption (Clarridge *et al.,* 2018). During ATI plasma HIV-1 RNA and HIV-1 DNA levels increased, but following re-initiation of ART and after a median of 363 days, HIV-1 DNA levels declined to pre-ATI levels.

Our study also found no significant correlation between CD4 percentage and absolute CD4 count at the end of study with duration of therapy interruption phase. This is not surprising as the CHER study reinitiated children based on CD4 count or other clinical criteria, which could have counteracted any effect of prolonged interruption on CD4 count. Similarly, another study found no significant lasting differences in pre-ATI and post-ATI CD4 count or CD4 percentage. Despite a transient decrease in CD4+ T cells during interruption upon re-initiation of treatment the levels of CD4+ T cells returned to pre-ATI levels (Clarridge *et al.*, 2018).

4.4 Summary of the scope of the study

4.4.1 Strengths and limitations of the study

When considering infants who were diagnosed and treated, shortly after birth (VEID cohort), infants were diagnosed in a public health setting and recruited only later in the study upon confirmation of positive HIV diagnosis. PBMC samples were not available for baseline HIV-1 DNA load testing and DBS samples were utilised. We attempted to normalize DBS samples by the amount of amplifiable cell equivalents, but due to variable cell recovery and DBS including polymorphonuclear cells we were not able to accurately quantify the amount of HIV-1 DNA in DBS. In addition, the lack of frequent sampling soon after therapy initiation prevented us from definitively determining whether the initial decay HIV-1 DNA decay was log-linear or biphasic. The iCAD assay

detects total HIV-1 DNA which mostly constitutes defective and unintegrated provirus (Bruner *et al.*, 2016). A study assessing the decay dynamics of integrated virus will be valuable because the population of cells may contain replication competent virus. Although there were a few limitations in our study, the integrase cell associated DNA assay used in this research project was ultrasensitive with a low limit of detection (3 copies per million cells), with a high success rate and reproducibility. As periods of poor adherence and virological failure could impact on HIV-1 decay, we only included 31 viraemia-suppressed CHER patients in the study of associations with HIV-1 DNA which limited the statistical power to investigate all associations with decay. We were also not able to study the effect of treatment interruption in isolation as only children who started ART early were interrupted.

Nevertheless, our study is one of very few longitudinal studies of HIV-1 DNA in children across a range of time points. This enabled us to investigate associates with the rate of decay and associations with decay over a period of ten years of antiretroviral therapy.

4.4.2 Future considerations

The mechanism of rapid HIV-1 DNA decay in children treated shortly after birth is not fully understood and requires further investigation. The proportion of HIV-1 infected cells in neonates with unintegrated versus integrated HIV-1 DNA is not known and the fraction of HIV-1 infected cells of different CD4 subsets and how that impacts on decay in early treated children is unknown. Assaying different subsets of CD4+ T cells in infants treated shortly after birth could provide insight into the lability of these immune cells and may provide insight into the mechanism of rapid decay, but this would remain challenging due to blood collection limits in infants. The proportion of proviruses that are intact or replication competent proviruses relative to defective proviruses in early treated infants is also not known. However, when using low HIV-1 DNA inputs, the most specific assay for replication competent HIV, the quantitative viral outgrowth assay (qVOA), has a very low yield in patients with low numbers of infected cells and its use had therefore not been feasible in these children. Future more sensitive molecular assays for intact proviruses may help to determine the true size of HIV-1 reservoirs and rate of decay of these true reservoirs. Analytical treatment interruption (ATI) studies are of growing importance especially with regards to studies investigating strategies to achieve HIV-1 remission. There is a lack of ATI studies in children. A comprehensive study investigating the effects of ATI on HIV-1 reservoirs (which include time to rebound) and immunological parameters in children would provide valuable information for the design of future clinical trials of curative interventions.

Chapter 5: Conclusion

In line with the aims and objectives of this study we accurately quantified total HIV-1 DNA decay using highly sensitive and reproducible assays; and using statistical methods we were able to assess predictors influencing the rate of HIV-1 DNA decay. In infants who were diagnosed and treated very shortly after birth, we observed rapid plasma suppression, in most, and total HIV-1 DNA became undetectable within four months of birth. We observed a faster decay rate in comparison to the reports of similar studies. Future research is required to accurately characterize the first phase of HIV-1 DNA decay in infants, but this would require the storage of PBMCs shortly after birth.

In our study we also observed that children who initiated ART at around five months had a significantly slower decay rate than those who initiated soon after birth. Furthermore, the strongest predictors of slower decay were high pre-treatment HIV-1 DNA levels and increase in HIV-1 DNA levels over the period of therapy interruption. However, we found no lasting effect on HIV-1 DNA decay due to interruption, but this could be because only children who started earlier were interrupted. Children who had a prolonged initial treatment period had a faster decay rate. Interruption, as implemented in the CHER study, without virological monitoring, is no longer considered. Nevertheless, the absence of the long-term effects of interruption on reservoirs and therapy provided for a sufficient duration after initial infection, is associated with a more rapid decay. This may suggest that children, who were treated early and for a sufficient time, may be safely interrupted as part of HIV cure studies, as long as they are monitored intensively to prevent HIV-1 reservoir re-seeding.

In conclusion, this study provided some of the first longitudinal data of HIV-1 DNA in children from a resource limited setting. We also showed that very early diagnosis and therapy initiation with adequate treatment adherence in the early stages of therapy may be valuable in limiting HIV-1 persistence.

Reference List

Abrams, E.J., Woldesenbet, S., Soares Silva, J., Coovadia, A., Black, V., Technau, K.-G. & Kuhn, L. 2017. Despite Access to Antiretrovirals for Prevention and Treatment, High Rates of Mortality Persist Among HIV-infected Infants and Young Children. *The Pediatric infectious disease journal*. 36(6):595–601.

Ananworanich, J., Puthanakit, T., Suntarattiwong, P., Chokephaibulkit, K., Kerr, S.J., Fromentin, R., Bakeman, W., Intasan, J., et al. 2014. Reduced markers of HIV persistence and restricted HIV-specific immune responses after early antiretroviral therapy in children. *AIDS*. 28(7):1015–1020.

Ananworanich, J., Dubé, K. & Chomont, N. 2015. How does the timing of antiretroviral therapy initiation in acute infection affect HIV reservoirs? *Curr Opin HIV AIDS*. 10(1):18–28.

Ananworanich, J., Chomont, N., Eller, L.A., Kroon, E., Tovanabutra, S., Bose, M., Nau, M., Fletcher, J.L.K., et al. 2016. HIV DNA Set Point is Rapidly Established in Acute HIV Infection and Dramatically Reduced by Early ART. *EBioMedicine*. 11:68–72.

Arts, E.J. & Hazuda, D.J. 2012. HIV-1 antiretroviral drug therapy. *Cold Spring Harbor Perspectives in Medicine*. 2(4).

Avettand-Fènoë, V., Hocqueloux, L., Ghosn, J., Cheret, A., Frange, P., Melard, A., Viard, J.P. & Rouzioux, C. 2016. Total HIV-1 DNA, a marker of viral reservoir dynamics with clinical implications. *Clinical Microbiology Reviews*. 29(4):859–880.

Bekker, L.-G., Alleyne, G., Baral, S., Cepeda, J., Daskalakis, D., Dowdy, D., Dybul, M., Eholie, S., et al. 2018. Advancing global health and strengthening the HIV response in the era of the Sustainable Development Goals: the International AIDS Society— Lancet Commission. *The Lancet*. 392(10144):312–358.

Bruner, K.M., Hosmane, N.N. & Siliciano, R.F. 2015. Towards an HIV-1 cure: Measuring the latent reservoir. *Trends in Microbiology*. 23(4):192–203.

Bruner, K.M., Murray, A.J., Pollack, R.A., Soliman, M.G., Laskey, S.B., Capoferri, A.A., Lai, J., Strain, M.C., et al. 2016. Defective proviruses rapidly accumulate during acute HIV-1 infection. *Nature Medicine*. 22(9):1043–1049.

Clarke, D.F., Yildirim, I. & Cooper, E.R. 2017. Rapid Initiation of Antiretrovirals in Two Newly Diagnosed HIV-infected Infants. *The Pediatric Infectious Disease Journal*. 36(1):61–62.

Clarridge, K.E., Blazkova, J., Einkauf, K., Petrone, M., Refsland, E.W., Justement, J.S., Shi, V., Huiting, E.D., et al. 2018. Effect of analytical treatment interruption and reinitiation of antiretroviral therapy on HIV reservoirs and immunologic parameters in infected individuals.

PLoS Pathogens. 14(1):1–16.

Coffin, J.M., Hughes, S.H. & Varmus, H.E. 1997. *Retroviruses*. Cold Spring Harbor Laboratory Press. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/21433340 [2019, January 10].

Connor, E.M., Sperling, R.S., Gelber, R., Kiselev, P., Scott, G., O'Sullivan, M.J., VanDyke, R., Bey, M., et al. 1994. Reduction of Maternal-Infant Transmission of Human Immunodeficiency Virus Type 1 with Zidovudine Treatment. *New England Journal of Medicine*. 331(18):1173–1180.

Cooper, E.R., Charurat, M., Mofenson, L., Hanson, I.C., Pitt, J., Diaz, C., Hayani, K., Handelsman, E., et al. 2002. Combination antiretroviral strategies for the treatment of pregnant HIV-1-infected women and prevention of perinatal HIV-1 transmission. *Journal of Acquired Immune Deficiency Syndromes*. 29(5):484–494.

Cotton, M.F., Violari, A., Otwombe, K., Panchia, R., Dobbels, E., Rabie, H., Josipovic, D., Liberty, A., et al. 2013. Early time-limited antiretroviral therapy versus deferred therapy in South African infants infected with HIV: Results from the children with HIV early antiretroviral (CHER) randomised trial. *The Lancet*. 382(9904):1555–1563.

Craigie, R. & Bushman, F.D. 2012. HIV DNA integration (2012).pdf. 1–18.

Deeks, S.G., Lewin, S.R., Ross, A.L., Ananworanich, J., Benkirane, M., Cannon, P., Chomont, N., Douek, D., et al. 2016. International AIDS Society global scientific strategy: towards an HIV cure 2016. *Nature Medicine*. 22(8):839–850.

Eriksson, S., Graf, E.H., Dahl, V., Strain, M.C., Yukl, S.A., Lysenko, E.S., Bosch, R.J., Lai, J., et al. 2013. Comparative Analysis of Measures of Viral Reservoirs in HIV-1 Eradication Studies. *PLoS Pathogens*. 9(2):e1003174.

Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, R.E., Quinn, T.C., Chadwick, K., et al. 1997. Identification of a Reservoir for HIV-1 in Patients on Highly Active Antiretroviral Therapy. *Science*. 278(November).

Foster, C., Pace, M., Kaye, S., Hopkins, E., Jones, M., Robinson, N., Mant, C., Cason, J., et al. 2017. Early antiretroviral therapy reduces HIV DNA following perinatal HIV infection. *Aids*. 31(13):1847–1851.

Fowler, M.G., Qin, M., Fiscus, S.A., Currier, J.S., Flynn, P.M., Chipato, T., McIntyre, J., Gnanashanmugam, D., et al. 2017. Benefits and Risks of Antiretroviral Therapy for Perinatal HIV Prevention. *Obstetrical and Gynecological Survey*. 72(3):143–145.

Goulder, P.J., Lewin, S.R. & Leitman, E.M. 2016. Paediatric HIV infection : the potential. *Nature Publishing Group*.

Gupta, R.K., Abdul-Jawad, S., McCoy, L.E., Mok, H.P., Peppa, D., Salgado, M., Martinez-Picado, J., Nijhuis, M., et al. 2019. HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell

transplantation. *Nature*. 4(March).

Henrich, T.J., Hanhauser, E., Marty, F.M., Sirignano, M.N., Keating, S., Lee, T.-H., Robles, Y.P., Davis, B.T., et al. 2014. Antiretroviral-Free HIV-1 Remission and Viral Rebound After Allogeneic Stem Cell Transplantation. *Annals of Internal Medicine*. 161(5):319.

Henrich, T.J., Hatano, H., Bacon, O., Hogan, L.E., Rutishauser, R., Hill, A., Kearney, M.F., Anderson, E.M., et al. 2017. HIV-1 persistence following extremely early initiation of antiretroviral therapy (ART) during acute HIV-1 infection: An observational study. *PLOS Medicine*. 14(11):e1002417.

Hill, A.L., Rosenbloom, D.I.S., Fu, F., Nowak, M.A. & Siliciano, R.F. 2014. Predicting the outcomes of treatment to eradicate the latent reservoir for HIV-1. *Proceedings of the National Academy of Sciences of the United States of America*. 111(37):13475–13480.

Ho, Y., Shan, L., Hosmane, N.N., Wang, J., Laskey, S.B., Daniel, I.S., Lai, J., Blankson, J.N., et al. 2013. Replication-competent non-induced proviruses in the latent reservoir increase barrier to HIV-1 cure. 155(3):540–551.

Hocqueloux, L., Avettand-fènoël, V., Jacquot, S., Prazuck, T., Legac, E., Mélard, A., Niang, M., Mille, C., et al. 2013. Long-term antiretroviral therapy initiated during primary HIV-1 infection is key to achieving both low HIV reservoirs and normal T cell counts. *Journal of Antimicrobial Chemotherapy*. 68(5):1169–1178.

Hong, F., Aga, E., Cillo, A.R., Yates, A.L., Besson, G., Fyne, E., Koontz, D.L., Jennings, C., et al. 2016. Novel assays for measurement of total cell-associated HIV-1 DNA and RNA. *Journal of Clinical Microbiology*. 54(4):902–911.

Huang, S.-H., Ren, Y., Thomas, A.S., Chan, D., Mueller, S., Ward, A.R., Patel, S., Bollard, C.M., et al. 2018. Latent HIV reservoirs exhibit inherent resistance to elimination by CD8+ T cells. *The Journal of Clinical Investigation*. 128(2):876–889.

Hütter, G., Nowak, D., Mossner, M., Ganepola, S., Müßig, A., Allers, K., Schneider, T., Hofmann, J., et al. 2009. Long-Term Control of HIV by *CCR5* Delta32/Delta32 Stem-Cell Transplantation. *New England Journal of Medicine*. 360(7):692–698.

Joint United Nations Program on HIV/AIDS. 2014. to help end the AIDS epidemic. *United Nations*.

Josefsson, L., King, M.S., Makitalo, B., Brannstrom, J., Shao, W., Maldarelli, F., Kearney, M.F., Hu, W.-S., et al. 2011. Majority of CD4+ T cells from peripheral blood of HIV-1-infected individuals contain only one HIV DNA molecule. *Proceedings of the National Academy of Sciences*. 108(27):11199–11204.

Kiselinova, M., De Spiegelaere, W., Buzon, M.J., Malatinkova, E., Lichterfeld, M. & Vandekerckhove, L. 2016. Integrated and Total HIV-1 DNA Predict Ex Vivo Viral Outgrowth.

PLoS Pathogens. 12(3).

Kuhn, L., Paximadis, M., Da Costa Dias, B., Loubser, S., Strehlau, R., Patel, F., Shiau, S., Coovadia, A., et al. 2018. Age at antiretroviral therapy initiation and cell-associated HIV-1 DNA levels in HIV-1 infected children. *PLoS ONE*. 13(4):1–14.

Laanani, M., Ghosn, J., Essat, A., Melard, A., Seng, R., Gousset, M., Panjo, H., Mortier, E., et al. 2015. Impact of the timing of initiation of antiretroviral therapy during primary HIV-1 infection on the decay of cell-associated HIV-DNA. *Clinical Infectious Diseases*. 60(11):1715–1721.

Lewis, J., Payne, H., Sarah Walker, A., Otwombe, K., Gibb, D.M., Babiker, A.G., Panchia, R., Cotton, M.F., et al. 2017. Thymic output and CD4 T-cell reconstitution in HIV-infected children on early and interrupted antiretroviral treatment: Evidence from the children with HIV early antiretroviral therapy trial. *Frontiers in Immunology*. 8(SEP).

Lilian, R.R., Kalk, E., Technau, K.-G. & Sherman, G.G. 2013. Birth Diagnosis of HIV Infection in Infants to Reduce Infant Mortality and Monitor for Elimination of Mother-to-child Transmission. *The Pediatric Infectious Disease Journal*. 32(10):1080–1085.

Luzuriaga, K. & Mofenson, L.M. 2016. Challenges in the Elimination of Pediatric HIV-1 Infection. *New England Journal of Medicine*. 374(8):761–770.

Luzuriaga, K., Tabak, B., Garber, M., Chen, Y.H., Ziemniak, C., McManus, M.M., Murray, D., Strain, M.C., et al. 2014. HIV type 1 (HIV-1) proviral reservoirs decay continuously under sustained virologic control in HIV-1-infected children who received early treatment. *The Journal of infectious diseases*. 210(10):1529–1538.

Luzuriaga, K., Gay, H., Ziemniak, C., Sanborn, K.B., Somasundaran, M., Rainwater-Lovett, K., Mellors, J.W., Rosenbloom, D., et al. 2015. Viremic Relapse after HIV-1 Remission in a Perinatally Infected Child. *New England Journal of Medicine*. 372(8):786–788.

Martin, G.E., Gossez, M., Williams, J.P., Stö Hr B, W., Meyerowitz, J., Leitman, E.M., Goulder, P., Porter, K., et al. 2017. Post-treatment control or treated controllers? Viral remission in treated and untreated primary HIV infection. *AIDS*. 31:477–484.

Martínez-Bonet, M., Puertas, M.C., Fortuny, C., Ouchi, D., Mellado, M.J., Rojo, P., Noguera-Julian, A., Muñoz-Fernández, M.A., et al. 2015. Establishment and Replenishment of the Viral Reservoir in Perinatally HIV-1-infected Children Initiating Very Early Antiretroviral Therapy. *Clinical Infectious Diseases*. 61(7):1169–1178.

Martinez, D.R., Permar, S.R. & Fouda, G.G. 2016 a. Contrasting Adult and Infant Immune Responses to HIV Infection and Vaccination.

Martinez, D.R., Permar, S.R. & Fouda, G.G. 2016 b. Contrasting Adult and Infant Immune Responses to HIV Infection and Vaccination. *Clinical and Vaccine Immunology*. 23(2):84–94.

MartinVA. n.d. *Mid-year population estimates 2017*. [Online], Available: www.statssa.gov.zainfo@statssa.gov.za [2019, January 15].

McManus, M., Mick, E., Hudson, R., Mofenson, L.M., Sullivan, J.L., Somasundaran, M. & Luzuriaga, K. 2016. Early combination antiretroviral therapy limits exposure to HIV-1 replication and cell-associated HIV-1 DNA levels in infants. *PLoS ONE*. 11(4):1–11.

Mikkelsen, E., Hontelez, J.A.C., Jansen, M.P.M., Bärnighausen, T., Hauck, K., Johansson, K.A., Meyer-Rath, G., Over, M., et al. 2017. Evidence for scaling up HIV treatment in sub-Saharan Africa: A call for incorporating health system constraints. *PLoS Medicine*. 14(2):1–5.

Pankau, M.D., Wamalwa, D., Benki-Nugent, S., Tapia, K., Ngugi, E., Langat, A., Otieno, V., Moraa, H., et al. 2018. Decay of HIV DNA in the Reservoir and the Impact of Short Treatment Interruption in Kenyan Infants. *Open Forum Infectious Diseases*. 5(1):13–16.

Papasavvas, E., Lada, S.M., Joseph, J., Yin, X., Liu, Q., Azzoni, L., Mounzer, K., Kostman, J.R., et al. 2018. Analytical antiretroviral therapy interruption does not irreversibly change preinterruption levels of cellular HIV. *Aids*. 32(13):1763–1772.

Persaud, D., Palumbo, P.E., Ziemniak, C., Hughes, M.D., Alvero, C.G., Luzuriaga, K., Yogev, R., Capparelli, E. V., et al. 2012. Dynamics of the resting CD4+ T-cell latent HIV reservoir in infants initiating HAART less than 6 months of age. *Aids*. 26(12):1483–1490.

Persaud, D., Gay, H., Ziemniak, C., Chen, Y.H., Piatak, M., Chun, T.-W., Strain, M., Richman, D., et al. 2013. Absence of Detectable HIV-1 Viremia after Treatment Cessation in an Infant. *New England Journal of Medicine*. 369(19):1828–1835.

Roider, J.M., Muenchhoff, M. & Goulder, P.J.R. 2016. Immune activation and paediatric HIV-1 disease outcome. *Current Opinion in HIV and AIDS*. 11(2):146–155.

Rouzioux, C. & Avettand-Fenoël, V. 2018. Total HIV DNA: A global marker of HIV persistence. *Retrovirology*. 15(1):1–7.

Sáez-Cirión, A., Bacchus, C., Hocqueloux, L., Avettand-Fenoel, V., Girault, I., Lecuroux, C., Potard, V., Versmisse, P., et al. 2013. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS pathogens*. 9(3):e1003211.

Schönland, S.O., Zimmer, J.K., Lopez-Benitez, C.M., Widmann, T., Ramin, K.D., Goronzy, J.J. & Weyand, C.M. 2003. Homeostatic control of T-cell generation in neonates. *Blood*. 102(4).

Sengupta, S. & Siliciano, R.F. 2018. Targeting the Latent Reservoir for HIV-1. *Immunity*. 48(5):872–895.

Sharaf, R.R. & Li, J.Z. 2017. The Alphabet Soup of HIV Reservoir Markers. *Current HIV/AIDS Reports*. 14(2):72–81.

Siliciano, R.F. & Greene, W.C. 2011. HIV latency. *Cold Spring Harbor Perspectives in Medicine*. 1(1):1–20.

Strain, M.C., Little, S.J., Daar, E.S., Havlir, D.V., Günthard, H.F., Lam, R.Y., Daly, O.A., Nguyen, J., et al. 2005. Effect of Treatment, during Primary Infection, on Establishment and Clearance of Cellular Reservoirs of HIV-1. *The Journal of Infectious Diseases*. 191(9):1410–1418.

Tagarro, A., Chan, M., Zangari, P., Ferns, B., Foster, C., De Rossi, A., Nastouli, E., Muñoz-Fernández, M.A., et al. 2018. Early and Highly Suppressive Antiretroviral Therapy Are Main Factors Associated With Low Viral Reservoir in European Perinatally HIV-Infected Children. *Journal of acquired immune deficiency syndromes (1999)*. 79(2):269–276.

Tobin, N.H. & Aldrovandi, G.M. 2013. Immunology of Pediatric HIV Infection. *Immunol Rev.* 254(1):143–169.

UNAIDS. 2018. *2017 Global HIV Statistics*. [Online], Available: aidsinfo.unaids.org.

Uprety, P., Chadwick, E.G., Rainwater-Lovett, K., Ziemniak, C., Luzuriaga, K., Capparelli, E. V., Yenokyan, G. & Persaud, D. 2015. Cell-Associated HIV-1 DNA and RNA Decay Dynamics during Early Combination Antiretroviral Therapy in HIV-1-Infected Infants. *Clinical Infectious Diseases*. 61(12):1862–1870.

Uprety, P., Patel, K., Karalius, B., Ziemniak, C., Chen, Y.H., Brummel, S.S., Siminski, S., Van Dyke, R.B., et al. 2017. Human immunodeficiency virus type 1 DNA decay dynamics with early, long-term virologic control of perinatal infection. *Clinical Infectious Diseases*. 64(11):1471–1478.

Violari, A., Cotton, M.F., Gibb, D.M., Babiker, A.G., Steyn, J., Madhi, S.A., Jean-Philippe, P. & McIntyre, J.A. 2008. Early Antiretroviral Therapy and Mortality among HIV-Infected Infants. *New England Journal of Medicine*. 359(21):2233–2244.

Violari, A., Cotton, M.F., Kuhn, L., Schramm, D.B., Paximadis, M., Loubser, S., Shalekoff, S., Da Costa Dias, B., et al. 2019. A child with perinatal HIV infection and long-term sustained virological control following antiretroviral treatment cessation. *Nature Communications*. 10(1):412.

Waitt, C.J., Garner, P., Bonnett, L.J., Khoo, S.H. & Else, L.J. 2015. Is infant exposure to antiretroviral drugs during breastfeeding quantitatively important? A systematic review and meta-analysis of pharmacokinetic studies. *Journal of Antimicrobial Chemotherapy*. 70(7):1928–1941.

WHO. 2015. Guidelines Guideline on When To Start Antiretroviral Therapy and on Pre-Exposure Prophylaxis for Hiv. *World Health Organization*. (September):78.

Van Zyl, G.U., Bedison, M.A., Van Rensburg, A.J., Laughton, B., Cotton, M.F. & Mellors, J.W.

2015. Early antiretroviral therapy in South African children reduces HIV-1-infected cells and cell-associated HIV-1 RNA in blood mononuclear cells. *Journal of Infectious Diseases*. 212(1):39–43.

Addenda

Addendum A

Addendum A1

A cell count was taken using a TC20™ Automated Cell Counter and the average count of live cells was used in the following calculations used to determine the amount of cryopreservation media was needed,

$$Average\ live\ count\ (cells/ml)\ x\ Volume\ of\ PBS\ used\ for\ cell\ count\ (ml) = Total\ live\ cells$$

$$Total\ Live\ cells\ (ml)\ \div\ 2.5x10^{6}\ cells/ml = Total\ volume\ of\ media$$

$$Total\ volume\ of\ media\ (ml)\ x\ 0.1 = DMSO\ (ml)$$

$$Total\ volume\ (ml) - DMSO\ volume(ml) = FBS\ (ml)$$

Addendum A2

Calculation used to determine the amount of Guanidinium hydrochloride and Proteinase K was needed to prepare the GuHCl/ProK solution:

$$\frac{50}{100} \times Total\ volume\ of\ GuHCl\ ProK\ solution\ = ProK\ (ml)$$

$$Total\ volume\ of\ GuHCl\ ProK - ProK\ (ml) = GuHCl\ (ml)$$

Calculation used to determine the amount of Guanidinium isothiocyanate and glycogen was needed to prepare the GuSCN/Gly solution:

$$\frac{30}{100} \times Total\ volume\ of\ GuSCN\ Gly\ solution = Gly\ (ml)$$

$$Total\ volume\ of\ GuSCN\ Gly - Gly\ (ml) = GuSCN\ ml)$$

Addendum A3

Forward primer: 5'-CCCTACAATCCCCAAAGTCA-3'

Reverse primer: 5'-CACAATCATCACCTGCCATC-3'

Addendum A4

Endemo DNA/RNA Copy Number Calculator (*http://endmemo.com/bio/dnacopynum.php*)

This online calculator was used to calculate the exact DNA copy number by inserting your DNA concentration, amplicon sequence and length in base pairs.

Addendum A5

CCR5 primers and probe

Forward primer: 5'- ATGATTCCTGGGAGAGACGC-3'

Reverse primer: 5'- AGCCAGGACGGTCACCTT-3'

Probe: 5'- /56-FAM/AACACAGCC/ZEN/ACCACCCAAGTGATCA/3IABkFQ/ -3'

HIV-1 integrase primers and probe

Forward primer: 5'-TTTGGAAAGGACCAGCCA-3'

Reverse primer: 5'- CCTGCCATCTGTTTTCCA-3'

Probe: 5'- /56-FAM/AAAGGTGAA/ZEN/GGGGCAGTAGTAATACA/3IABkFQ/-3'

Addendum B

Addendum B1

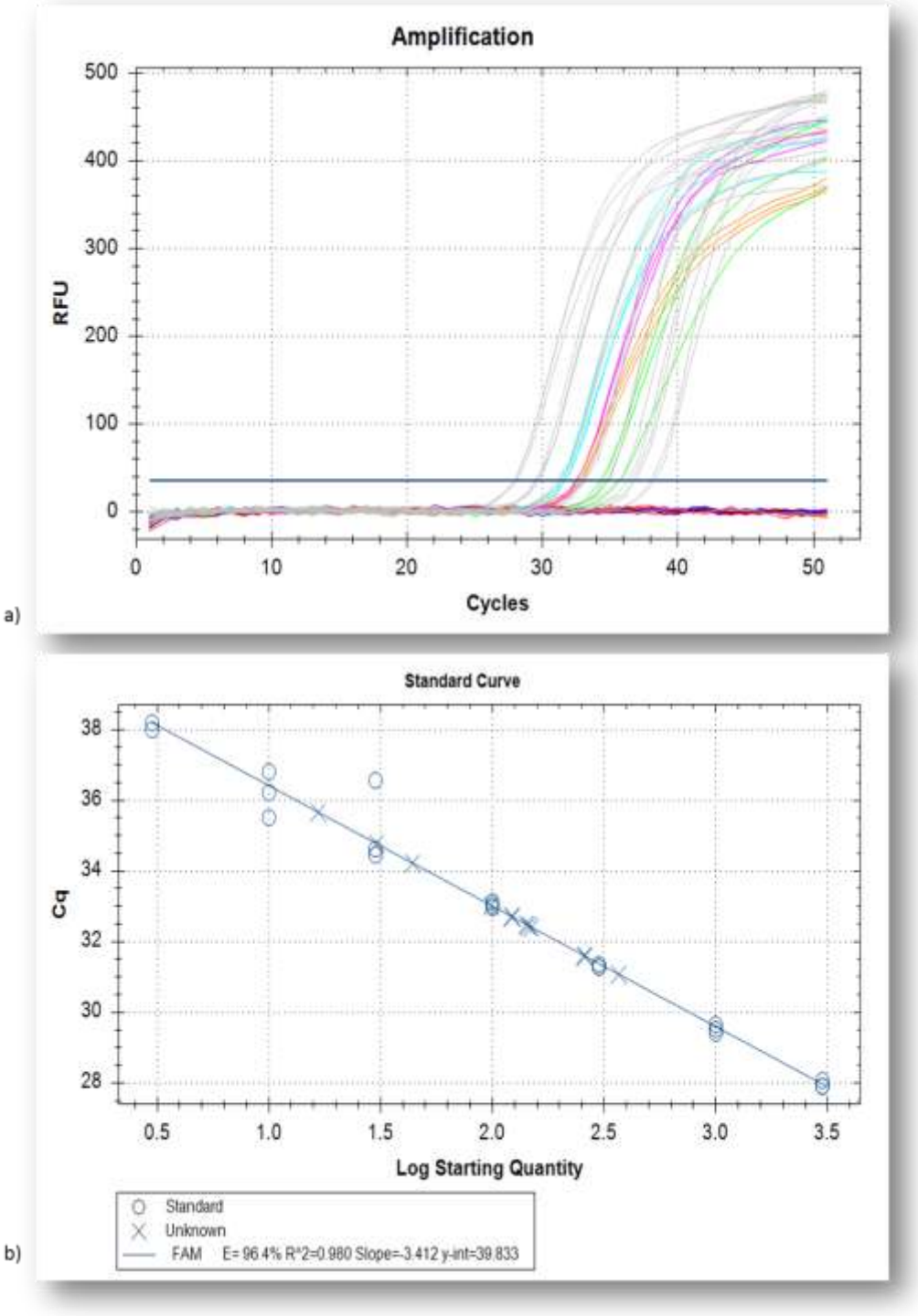
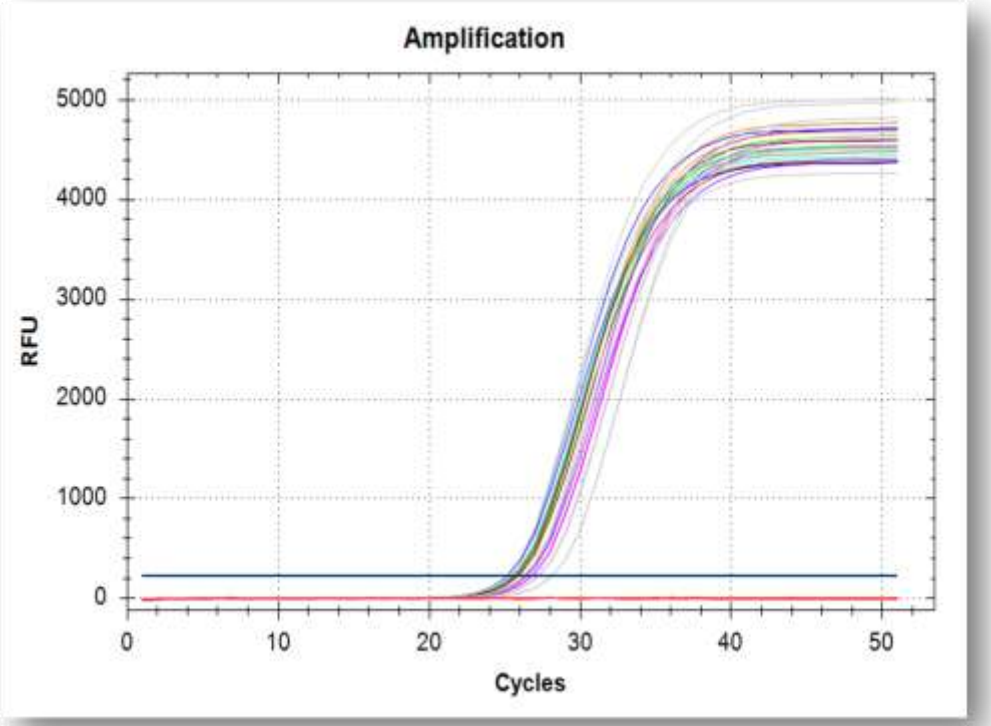


Figure B1.1 Example of a successful qPCR experiment quantifying total HIV-1 DNA in one of the VEID participants. a) Amplification curve, the HIV-1 integrase standard is indicated in grey and the patient sample at different time points are indicated in colour. b) Standard curve of the same qPCR experiment which indicates an acceptable efficiency and R^2 value. The standard is denoted by an open circle and the samples are denoted with an “x”.

a)



b)

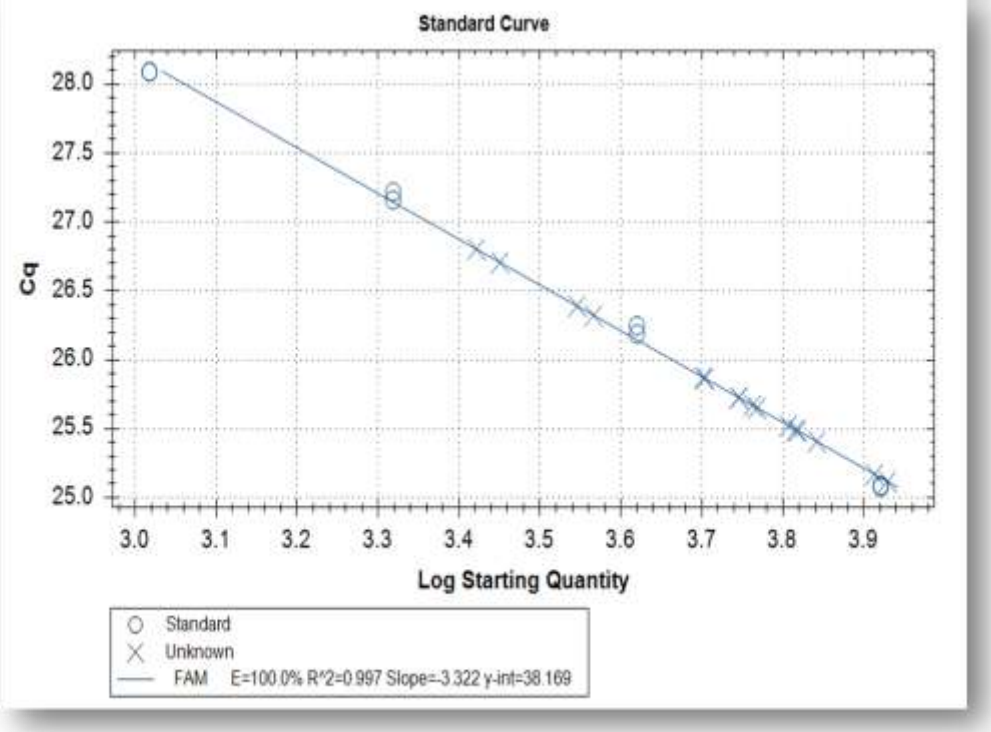


Figure B1.2 In relation to the above figure (B1.1.) during the same qPCR experiment (VEID participant) CCR5 is quantified and used to measure the total number of cells being assessed. a) Amplification curve of the CCR5 standard (TaqMan Human genomic DNA) is indicated in grey and patient samples at different time points are indicated in colour. b) Standard curve of the same qPCR experiment which indicates an acceptable efficiency and R^2 values. The standard is denoted by an open circle and the samples are denoted with an “x”.

Addendum B2

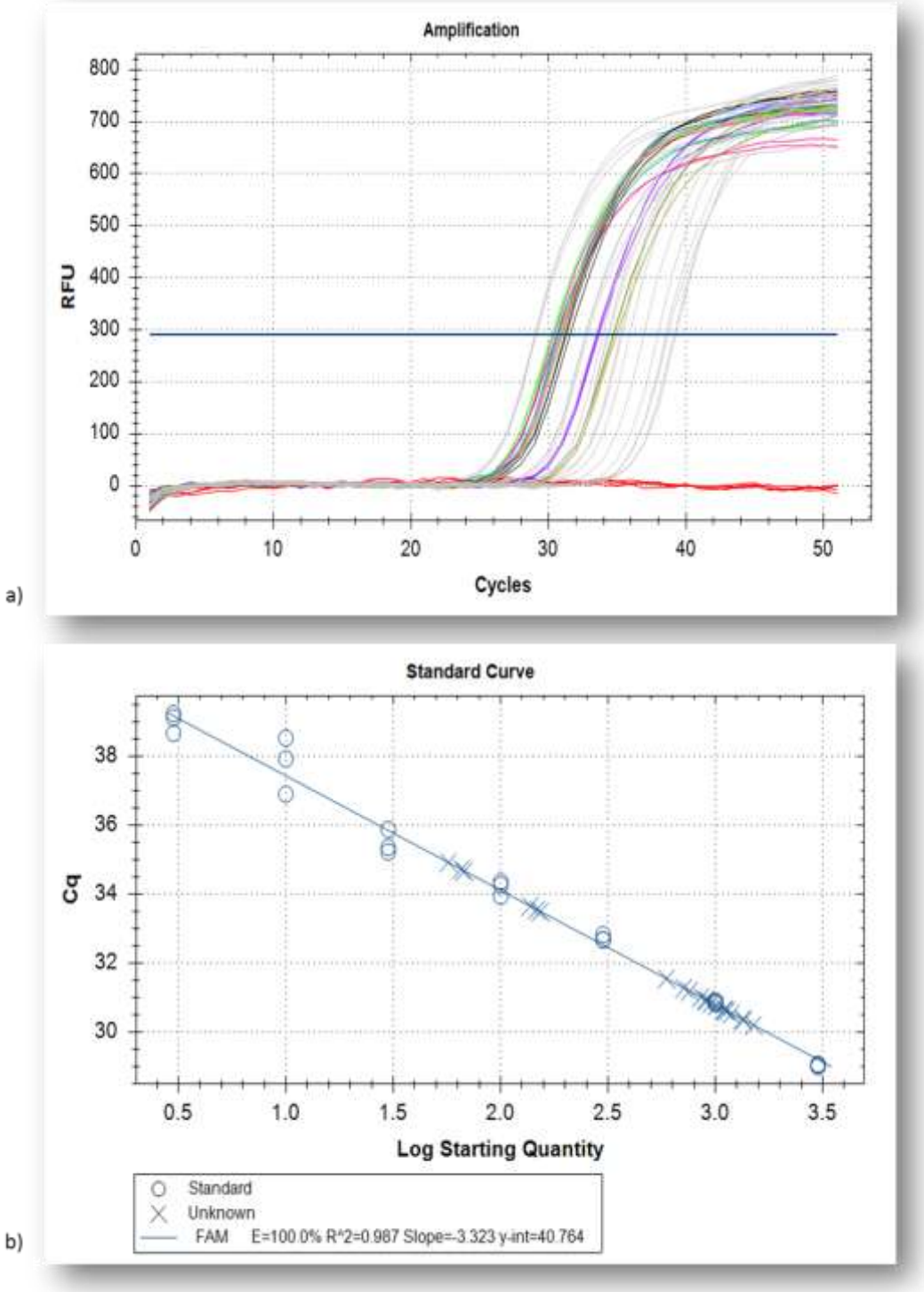
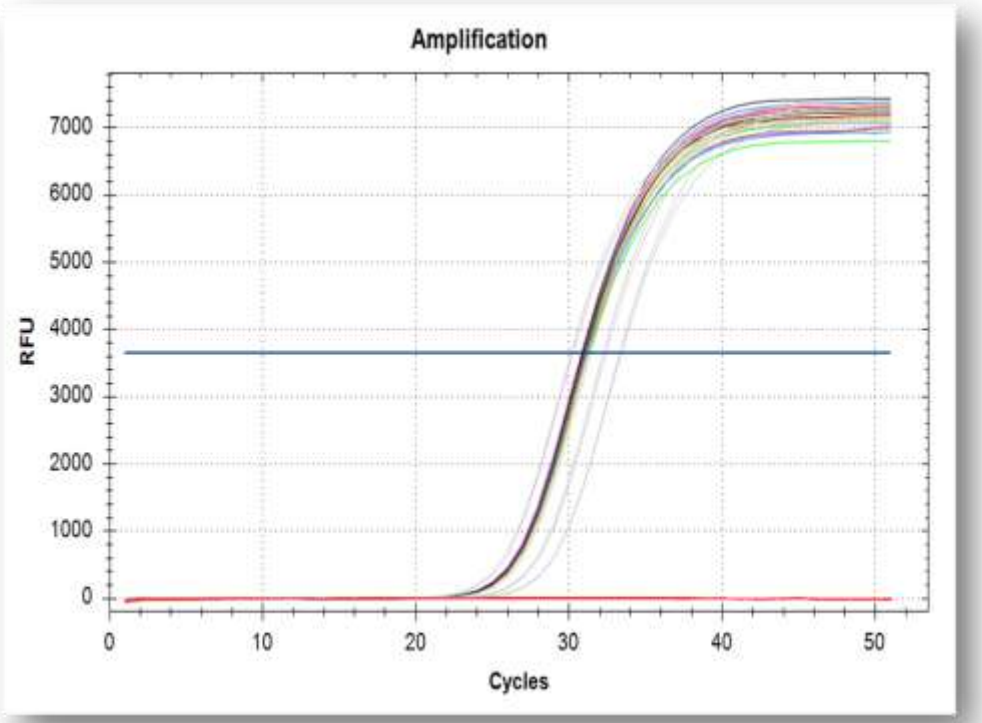


Figure B2.1 Example of a successful qPCR experiment quantifying total HIV-1 DNA in one of the CHER/Post-CHER participants. a) Amplification curve, the HIV-1 integrase standard is indicated in grey and the patient sample at different time points are indicated in colour. b) Standard curve of the same qPCR experiment which indicates an acceptable efficiency and R^2 value. The standard is denoted by an open circle and the samples are denoted with an “x”.

a)



b)

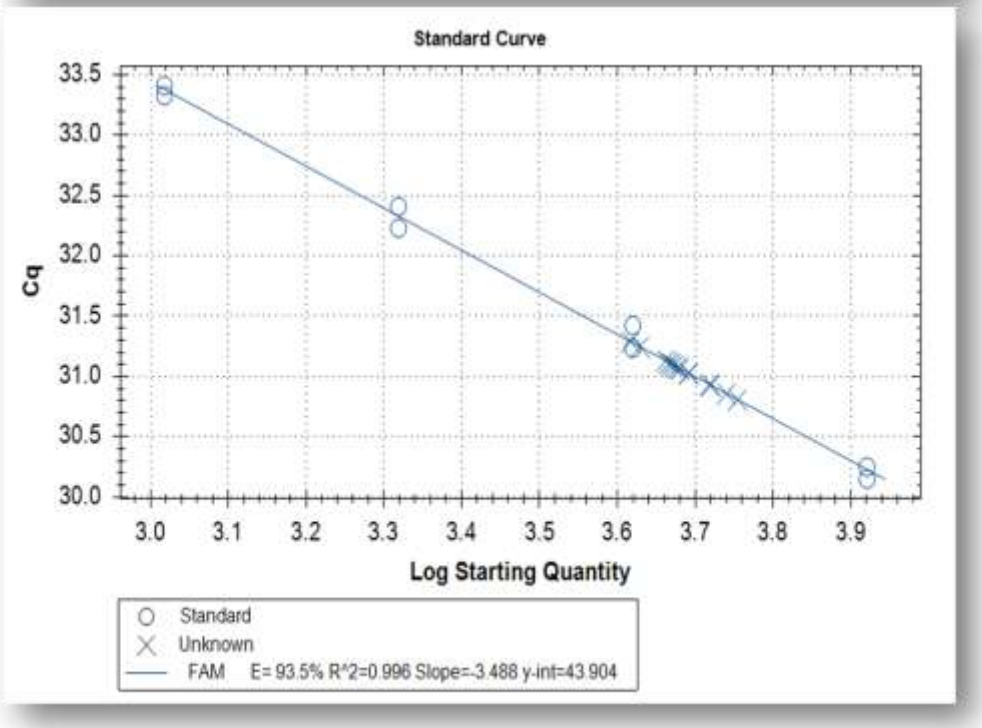


Figure B2.2 In relation to the above figure (B2.1) during the same qPCR experiment (CHER/Post-CHER participant) CCR5 is quantified and used to measure the total number of cells being assessed. a) Amplification curve of the CCR5 standard (TaqMan Human genomic DNA) is indicated in grey and patient samples at different time points are indicated in colour. b) Standard curve of the same qPCR experiment which indicates an acceptable efficiency and R^2 value. The standard is denoted by an open circle and the samples are denoted with an “x”.

“Success is not final, failure is not fatal: it is the courage to continue that counts.”

Winston Churchill